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Na^+-Cl^--GLUTAMATE COTRANSPORT BY LOBSTER HEPATOPANCREATIC BRUSH BORDER MEMBRANE VESICLES

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SUMMARY

L-[^{3}H]glutamate uptake by lobster hepatopancreatic brush border membrane vesicles, formed by a magnesium precipitation technique, was stimulated by a transmembrane NaCl gradient (o > i), but not by identical gradients of KCl, TMA-Cl, NaSCN or $NaNO_3$, suggesting that the amino acid transfer depends specifically upon both Na^+ and Cl^-. In the presence of a NaCl gradient (o > i), glutamate uptake was strongly dependent upon bilateral pH and increased markedly as pH was lowered from 7·0 to 4·0. NaCl-dependent L-[^{3}H]glutamate uptake was not trans-stimulated by preloading vesicles with K^+. At pH 5·0, NaCl-dependent glutamate uptake occurred by an electroneutral process, whereas Na^+-dependent D-[^{3}H]glucose uptake at this pH was electrogenic. L-[^{3}H]glutamate influx exhibited both carrier-mediated and apparent diffusional transport components. Decreasing pH had no significant effect on glutamate influx (K_t), but tripled both maximal carrier-mediated entry rate (J_M) and the apparent diffusional permeability (P) of the membrane to this compound. L-[^{3}H]glutamate influx was strongly trans-stimulated by vesicles preloaded with L- and D-glutamate, L- and D-aspartate, L-cysteate, L-tyrosine and L-asparagine, but not by vesicles preloaded with L-leucine, L-glutamine, L-proline, L-alanine or L-lysine. L-[^{3}H]glutamate influx at pH 4·0 was a hyperbolic function of both external Na^+ and Cl^- concentrations at fixed concentrations of the respective counterions, suggesting cotransport between the amino acid and the two ions with a stoichiometry of 1 Na^+ : 1 Cl^- : 1 glutamate.

INTRODUCTION

Amino acid transport across epithelial membranes occurs by a variety of carrier processes that illustrate various degrees of substrate specificity and are either sodium-dependent or sodium-independent (Stevens, Ross & Wright, 1982). While several distinct membrane carriers have been described for neutral amino acids with broadly overlapping specificities (Preston, Schaffer & Curran, 1974), the transport of charged amino acids, either cationic or anionic, appears to be largely restricted to their own respective transfer systems. In vertebrate intestinal and renal epithelia, the

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acidic amino acids L-glutamate and L-aspartate appear to be transported across membranes by a Na^+-dependent system that accepts a narrow range of substrate configurations (Schultz, Yu-tu, Alvarez & Curran, 1970; Lerner & Steinke, 1977; Wëiss, McNamara, Pepe & Segal, 1978; Schneider & Sacktor, 1980; Schneider, Hammerman & Sactor, 1980). The Na^+-glutamate cotransport system is electroneutral in the absence of internal K^+ (Schneider *et al.* 1980; Corcelli, Prezioso, Palmieri & Storelli, 1982) but may carry a net charge in the presence of trans concentrations of K^+ (Burckhardt, Kinne, Stange & Murer, 1980). Furthermore, external Cl^- may have an undefined role in the transfer of this amino acid (Corcelli & Storelli, 1983).

In crustaceans the hepatopancreas, a large multilobate diverticulum of the stomach lined by a single layer of epithelium, is a major centre for nutrient absorption, and its epithelial brush border membranes exhibit Na^+-dependent (Ahearn, Grover & Dunn, 1985) and Na^+-independent (Ahearn *et al.* 1986; Ahearn & Clay, 1987) carrier mechanisms for sugars and amino acids. This study examines the nature of L-glutamate transport by hepatopancreatic brush border membranes, focusing upon the specific roles of Na^+, K^+ and Cl^- in amino acid transfer. Our findings show that the uptake of glutamate is coupled with that of both Na^+ and Cl^-, and that there is apparently no stimulatory effect of internal K^+ on amino acid transport.

MATERIALS AND METHODS

Live intermoult Atlantic lobsters (*Homarus americanus* Milne Edwards; 0·5 kg each) were purchased from commercial dealers in Hawaii and maintained unfed at 10°C for up to 1 week in filtered sea water. Hepatopancreatic brush border membrane vesicles (BBMV) were prepared from fresh tissue removed from individual animals. Each batch of membranes was produced from a single organ (approx. 25 g fresh mass) using a magnesium precipitation technique described previously (Ahearn *et al.* 1985, 1986; Ahearn & Clay, 1987). Purification of a final membrane sample was assessed by comparing enzyme activities of this pellet with those of the original tissue homogenate. These comparisons showed final pellet enrichments of alkaline phosphatase, Na^+/K^+-ATPase and cytochrome *c* oxidase of 15·3-, 1·0- and 0·2-fold, respectively (Ahearn *et al.* 1985), suggesting that this method produced membranes which were rich in brush borders and reduced in contamination from the basolateral membrane or membranes from cellular organelles such as mitochondria.

Transport studies using hepatopancreatic BBMV were generally conducted at 15°C using a temperature-controlled water bath and the Millipore filtration technique developed by Hopfer, Nelson, Perrotto & Isselbacher (1973). Two types of transport experiments were performed. In long-term incubations a volume of membrane vesicles (e.g. 20 μl) was added to a volume of radiolabelled medium (e.g. 160 μl) containing L-[3,4-^{3}H]glutamate (ICN Radiochemicals). After incubation (15 s, 1, 2, 5, 10, 20, 90 min; longer equilibrium intervals of 120 or 180 min were used in some cases), a known volume of this reaction mixture (20 μl) was withdrawn and

plunged into 1·5 ml of ice-cold stop solution (composition generally the same as the respective outside medium except lacking the isotope label) to stop the uptake process. The resulting suspensions were rapidly filtered through Millipore filters (0·65 μm) to retain the vesicles and washed with another 10 ml of stop solution. Filters were then added to Beckman Ready-Solv HP scintillation cocktail and counted for radioactivity in a Beckman LS-8100 scintillation counter.

In short-term incubations, 5 μl of membrane suspension was mixed for predetermined time intervals with 45 μl of buffer containing the isotope at 23 °C using a rapid-exposure uptake apparatus (Inovativ Labor AG, Adliswil, Switzerland). Following isotope incubation, an ice-cold stop solution was injected into the membrane–isotope mixture. Vesicles in the stop solution were then treated as described above for long-term incubations.

The composition of the intravesicular medium was established by resuspending the penultimate membrane pellet in the appropriate internal solution with a Potter–Elvehjem homogenizer and allowing this mixture to stand on ice for 90 min prior to the final 30-min high-speed centrifugation. Vesicles therefore had normally been incubated in internal media for at least 120 min before a transport experiment was initiated.

Glutamate uptake values were expressed as pmol (using specific activity of L-[^{3}H]glutamate in the medium) per mg protein (Bio Rad protein assay) per filter. Each experiment was generally repeated two or three times using membranes prepared from different animals. Similar experimental findings were consistently obtained in repeated experiments. Within a given experiment, 3–5 replicates were used and the experimental scatter was generally around 10–15 %. Throughout this study, mean values are given with their standard errors.

CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and other reagent grade chemicals were obtained from Sigma Chemical Co.

RESULTS

Effects of inwardly directed cation gradients on glutamate uptake

The effects of inwardly directed cation gradients on the time course of uptake of 0·05 mmol l^{-1} L-[^{3}H]glutamate by lobster BBMV were examined by loading the vesicles with 200 mmol l^{-1} mannitol at pH 5·0 and incubating them in media at the same pH containing 100 mmol l^{-1} NaCl, KCl or TMA-Cl (tetramethylammonium chloride; Aldrich Chemical Co.) and the labelled amino acid. Fig. 1 indicates that vesicles incubated in 100 mmol l^{-1} NaCl exhibited significantly ($P < 0·05$) greater apparent amino acid influx rates (15-s uptake) than those exposed to the other two media. In addition, a marked glutamate uptake overshoot occurred in the former medium (the intravesicular amino acid concentration transiently exceeded that at equilibrium, 180 min, by a factor of three) but not in the latter two media. These results suggest that a transmembrane Na^+ gradient is required for concentrative uptake of L-[^{3}H]glutamate at pH 5·0 and that the requirement shows a strong specificity for this cation.

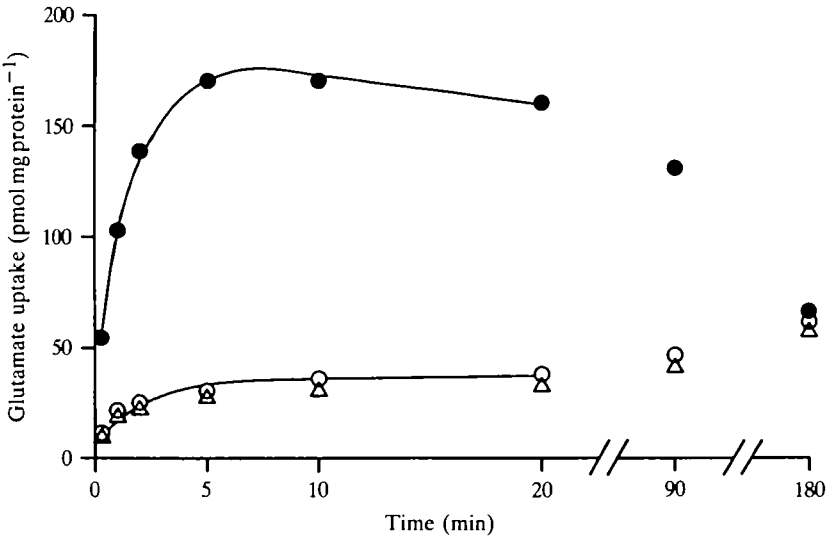

Fig. 1. Effects of inwardly directed cation gradients on the time course of uptake of 0·05 mmol l^{-1} L-[^{3}H]glutamate by lobster brush border membrane vesicles. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 5·0 and were incubated in media at the same pH containing 100 mmol l^{-1} NaCl (●), KCl (○) or TMA-Cl (△) and the labelled amino acid. Mes-Tris was used as the buffer in each medium.

pH dependence of L-[^{3}H]glutamate uptake

The effect of variations in bilateral pH on uptake of 0·05 mmol l^{-1} L-[^{3}H]glutamate in the presence of an inwardly directed NaCl gradient was assessed by loading vesicles with 200 mmol l^{-1} mannitol at pH 4·0, 5·0, 7·0 and 8·0 and incubating them in media of the same respective pH values containing 100 mmol l^{-1} NaCl and the labelled amino acid. Fig. 2 indicates that only at pH 4·0 and 5·0 was there concentrative glutamate accumulation which exceeded that at the respective 90-min uptake values. Uptake values at 180 min of incubation were not significantly different from one another, suggesting that at this time intravesicular isotope was in equilibrium with label in the respective external medium (as described in Fig. 1) and that similar degrees of vesiculation occurred at each pH. In addition, apparent 15-s amino acid influxes significantly ($P < 0·05$) increased as pH was lowered from 8·0 to 4·0. At pH 7·0 and 8·0, glutamate uptake was hyperbolic in character and slowly increased to maximal values at 90 min. These data suggest that, in the presence of an inwardly directed NaCl gradient, L-[^{3}H]glutamate transport is stimulated by a drop in pH and that transient vesicular accumulation of the amino acid only occurs when the pH is acidic.

Effects of inwardly directed anion gradients on glutamate uptake

Fig. 3 shows the result of an experiment designed to illustrate the anion dependence of glutamate transport at acidic pH. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 and were incubated in media of the same pH, but which contained 100 mmol l^{-1} NaCl, NaSCN, $NaNO_3$ or 200 mmol l^{-1} mannitol,

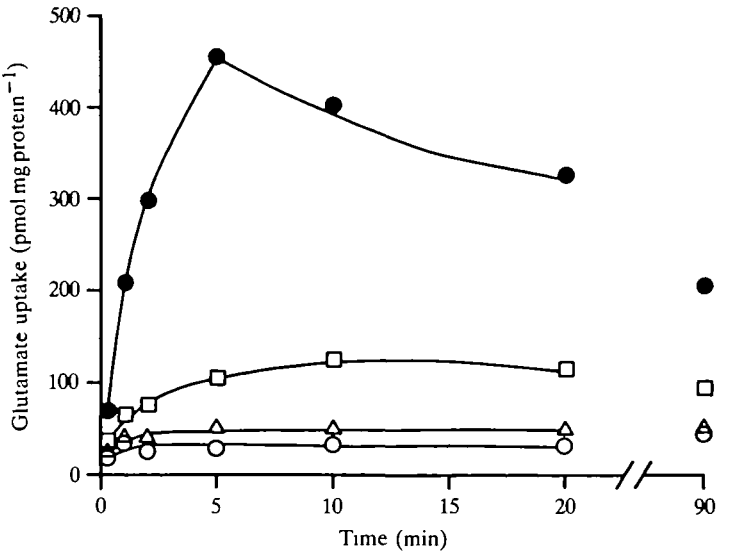

Fig. 2. Effects of bilateral pH on the time course of glutamate uptake by lobster brush border membrane vesicles. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 (●), 5·0 (□), 7·0 (△) or 8·0 (○) and were incubated in media at the same respective pH containing 100 mmol l^{-1} NaCl and 0·05 mmol l^{-1} L-[^{3}H]glutamate. Buffers used in all media were either Hepes-Tris or Mes-Tris.

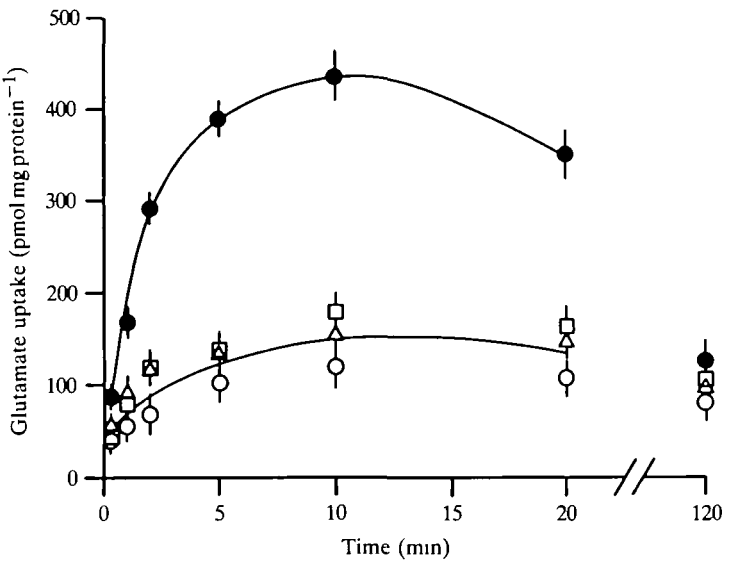

Fig. 3. Effects of inwardly directed anion gradients on the time course of glutamate uptake by lobster brush border membrane vesicles. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 (Mes-Tris buffer) and were incubated in media of the same pH containing 100 mmol l^{-1} NaCl (●), NaSCN (○), $NaNO_3$ (□) or 200 mmol l^{-1} mannitol (△), and 0·05 mmol l^{-1} L-[^{3}H]glutamate. Error bars indicate ±S.E.M.

and 0·05 mmol l^{-1} L-[^{3}H]glutamate. Transient L-[^{3}H]glutamate accumulation against a concentration gradient only occurred in the presence of an inwardly directed NaCl gradient, all other conditions led to slow hyperbolic uptake of the amino acid. L-[^{3}H]glutamate uptakes in $NaNO_3$- and NaSCN-containing media were not significantly different ($P > 0{\cdot}05$) from that in mannitol over the entire time course, suggesting that neither salt produced a significant stimulation of amino acid uptake. In addition, apparent L-[^{3}H]glutamate influx at 15 s in NaCl-containing medium was significantly ($P < 0{\cdot}05$) greater than amino acid influx in all other media. These results, and those illustrated in Fig. 1, suggest that concentrative transport of L-[^{3}H]glutamate by hepatopancreatic BBMV at acidic pH requires inwardly directed gradients of both Na^+ and Cl^- and that, in the absence of either ion, transient accumulation against a concentration gradient is abolished and apparent unidirectional transport to the vesicular interior is markedly reduced.

Effect of an outwardly directed potassium gradient on NaCl-dependent glutamate transport

To determine whether an outwardly directed K^+ gradient enhanced glutamate uptake in the presence of inwardly directed gradients of Na^+ and Cl^-, vesicles were preloaded with 200 mmol l^{-1} mannitol, 100 mmol l^{-1} KNO_3 or 100 mmol l^{-1} KCl at pH 4·0 and were incubated in media containing 0·05 mmol l^{-1} L-[^{3}H]glutamate and either 100 mmol l^{-1} NaCl or 100 mmol l^{-1} $NaNO_3$ at the same pH. Fig. 4 shows that L-[^{3}H]glutamate uptake was strongly stimulated in the presence of an inwardly directed gradient of NaCl as indicated earlier (Figs 1, 2, 3). When an outwardly directed gradient of KNO_3 or KCl was added to inwardly directed NaCl or $NaNO_3$ gradients, the uptake of the amino acid was significantly reduced. The rate of uptake

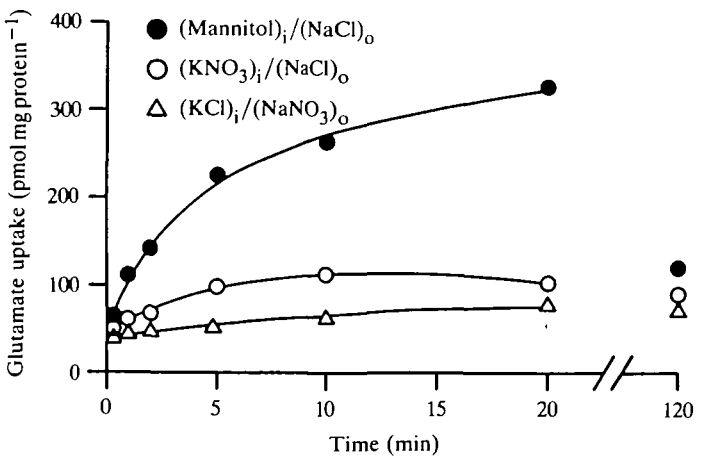

Fig. 4. Effects of an outwardly directed K^+ gradient on the time course of NaCl-dependent glutamate uptake by lobster brush border membrane vesicles. Vesicles were loaded with 200 mmol l^{-1} mannitol, 100 mmol l^{-1} KNO_3 or 100 mmol l^{-1} KCl at pH 4·0 (Mes-Tris buffer) and were incubated in media at the same pH which contained 100 mmol l^{-1} NaCl or 100 mmol l^{-1} $NaNO_3$, and 0·05 mmol l^{-1} L-[^{3}H]glutamate.

of L-[^{3}H]glutamate was lowest in vesicles having combined outward gradients of K^+ and Cl^- at the same time as an inward gradient of Na^+. These results provide further support for the dependence of L-[^{3}H]glutamate uptake on both external Na^+ and Cl^-. In addition, Fig. 4 clearly shows that intravesicular K^+ or KCl do not have a stimulating effect on the entry of the amino acid, but may even inhibit the transfer of the organic solute in some undetermined manner.

Evidence for electroneutral glutamate transport

Previous work has indicated that D-[^{3}H]glucose transport by lobster hepatopancreatic BBMV is Na^+-dependent and electrogenic in the presence of an inwardly directed Na^+ gradient, transferring a net positive charge to the vesicular interior during sugar accumulation (Ahearn *et al.* 1985). The simultaneous presence of an inwardly directed gradient of a permeable anion stimulates Na^+-dependent glucose uptake by this preparation due to electrical coupling between the transported solutes. In short-circuited vesicles, Na^+-dependent uptake of glucose in the presence of an inwardly directed anion gradient is significantly reduced.

To define the electrogenic nature of NaCl-dependent L-[^{3}H]glutamate transport, the effects of the protonophore, CCCP, on the uptake of glutamate in the presence of an inwardly directed NaCl gradient at pH 5·0 were compared to the effects of the drug on Na^+-dependent glucose transport in the same preparation. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 5·0 and were incubated in media of the same pH containing 100 mmol l^{-1} NaCl and either 0·05 mmol l^{-1} D-[^{3}H]glucose or 0·05 mmol l^{-1} L-[^{3}H]glutamate. One group of vesicles for each organic solute was preincubated in 50 μmol l^{-1} CCCP for 30 min prior to experimentation. Fig. 5 shows

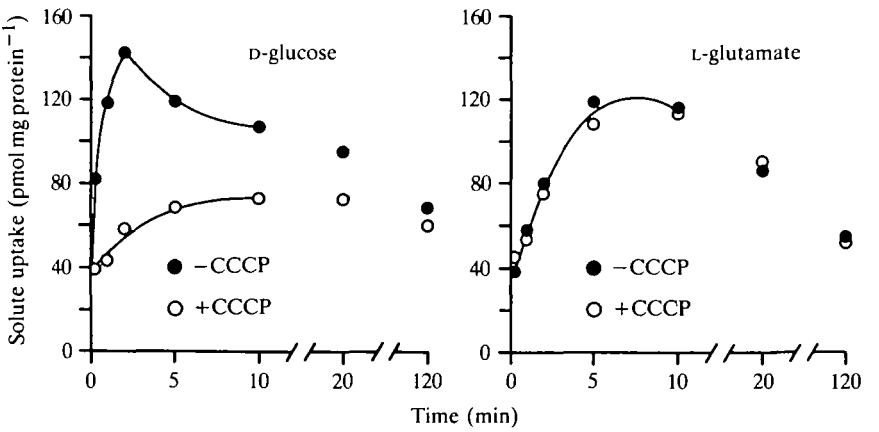

Fig. 5. Effects of the protonophore, CCCP, on the time course of uptake of 0·05 mmol l^{-1} D-[^{3}H]glucose or L-[^{3}H]glutamate by lobster brush border membrane vesicles. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 5·0 (Mes-Tris buffer) and were incubated in media of the same pH containing 100 mmol l^{-1} NaCl and either radiolabelled solute. One group of vesicles for each organic solute was preincubated in 50 μmol l^{-1} CCCP for 30 min before the experiment.

that, in the absence of the ionophore, D-[^{3}H]glucose uptake exhibited a strong NaCl dependency which was abolished when the vesicles were short-circuited. In contrast, CCCP had no significant effect ($P > 0{\cdot}05$) on L-[^{3}H]glutamate uptake. These results suggest that NaCl-dependent L-glutamate transport is electroneutral, whereas Na^+-dependent glucose uptake is strongly electrogenic.

Additional evidence for a contrast in the electrogenic nature of D-glucose and L-glutamate transport relates to the relative effects of a transmembrane gradient of NaSCN on the time course of uptake of these two organic solutes. As mentioned previously, Ahearn *et al.* (1985) indicated that the greatest D-glucose influx and overshoot by hepatopancreatic BBMV occurred in mannitol-loaded vesicles incubated in 100 mmol l^{-1} NaSCN. Less transport of the sugar took place in media containing NaCl, $NaSO_4$ or sodium gluconate. Because membranes are generally more permeable to SCN^- than to the other anions used in this experiment, it was concluded that the stimulatory effect of SCN^- was due to electrical coupling between the anion and sodium-D-glucose cotransport. In the present investigation, L-[^{3}H]glutamate uptake was not stimulated by a transmembrane NaSCN gradient of identical magnitude to that used in the D-glucose experiment described above (Fig. 3). These results imply the lack of electrical coupling between the transfer of the anion (SCN^-) and the sodium-dependent amino acid transport process, and provide support for an electroneutral transfer of L-glutamate in the presence of an inward gradient of NaCl.

Kinetics of glutamate influx

The time course of L-[^{3}H]glutamate uptake by lobster BBMV was examined at very short intervals (2, 4, 6, 8, 10, 15 and 20 s) using a rapid uptake apparatus to establish an accurate assessment of initial entry rates of 0·05 mmol l^{-1} L-[^{3}H]glutamate at pH 4·0, 5·0 and 6·0. Vesicles were loaded with 200 mmol l^{-1} mannitol at each pH and were incubated in media containing 100 mmol l^{-1} NaCl and 0·05 mmol l^{-1} L-[^{3}H]glutamate at pH 4·0, 5·0 and 6·0. Triplicate samples were obtained for each time. Uptake of 0·05 mmol l^{-1} L-glutamate by BBMV was a linear function of time from 2 to 20 s of incubation at each pH value (Fig. 6). The time course of uptake of 5·0 mmol l^{-1} L-glutamate had a similar pattern, while that of 20·0 mmol l^{-1} L-glutamate uptake was linear for only 10 s. Straight lines drawn through the data in Fig. 6 were based on linear regression analysis and provide an estimate of unidirectional L-glutamate transfer (slope) and L-glutamate binding (extrapolated vertical intercept). L-glutamate binding values, determined from calculated vertical intercepts at each pH, were not significantly different from one another ($P > 0{\cdot}05$) or from those obtained using vesicles injected into ice-cold (0°C) uptake medium and then rapidly filtered ('blank uptake values'). Influxes of 0·05 mmol l^{-1} L-[^{3}H]glutamate (J_{oi}) at pH 4·0, 5·0 and 6·0 were $1{\cdot}3 \pm 0{\cdot}2$, $0{\cdot}6 \pm 0{\cdot}1$ and $0{\cdot}3 \pm 0{\cdot}04$ pmol mg protein^{-1} s^{-1}, respectively. Significant ($P < 0{\cdot}05$) L-[^{3}H]glutamate binding to vesicles occurred at all pH values in 0·05 mmol l^{-1} glutamate and

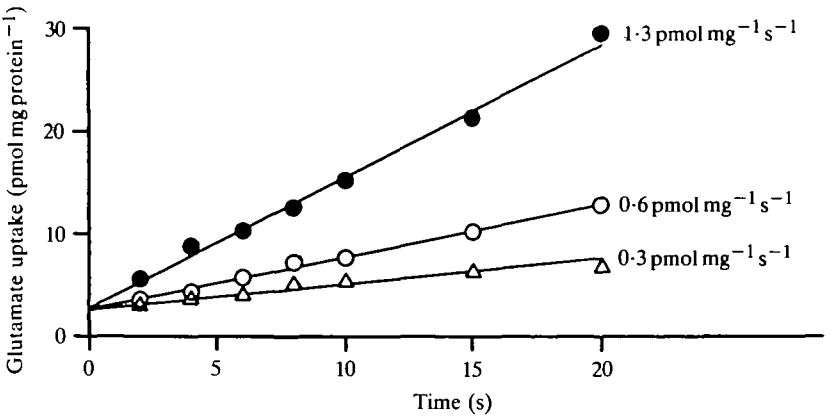

Fig. 6. Effects of bilateral pH on the time course of uptake of 0·05 mmol l^{-1} L-[^{3}H]glutamate at very short time intervals (2, 4, 6, 8, 10, 15, 20 s). Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4 (●), 5 (○) or 6 (△) and were incubated in media of the same pH containing 100 mmol l^{-1} NaCl and the radiolabelled amino acid. Straight lines drawn through the data were obtained by linear regression analysis and calculated slopes are presented.

represented approximately 16 and 50 % of total isotope uptake at 10 s for pH 4·0 and 6·0 media, respectively. Similar percentages of total uptake due to binding occurred in 5·0 mmol l^{-1} L-glutamate for both pH 4·0 and 6·0. Therefore, these estimated values of surface binding were used in subsequent 10-s uptake (influx) measurements of this amino acid at both pH values over an L-glutamate concentration range of 0·05 to 20 mmol l^{-1}.

Fig. 7 shows the effects of pH on L-[^{3}H]glutamate influx from incubation medium to vesicular interior determined in the manner described in Fig. 6 for external glutamate concentrations ranging from 0·05 to 20 mmol l^{-1}. In all cases vesicles were loaded with 200 mmol l^{-1} mannitol at the respective pH and were incubated for 10 s in media containing 100 mmol l^{-1} NaCl and the selected amino acid concentration. Over this concentration range, influx was a curvilinear function of external amino acid concentration at both pH values and could be described as the sum of at least two independent processes operating simultaneously: (1) a Michaelis–Menten carrier mechanism illustrating saturation kinetics, and (2) a linear entry system with a rate that was proportional to the external amino acid concentration and probably represented simple diffusion. At either pH these two processes operating together can be described by the equation:

$$J_{oi} = \frac{J_M[G]}{K_t + [G]} + P[G],$$

where J_{oi} is total L-[^{3}H]glutamate influx in pmol mg protein^{-1} s^{-1}, J_M is maximal carrier-mediated influx, K_t is the L-[^{3}H]glutamate concentration resulting in half-maximal influx, [G] is the external amino acid concentration and P is the rate constant of the linear process which can be defined as an 'apparent diffusional

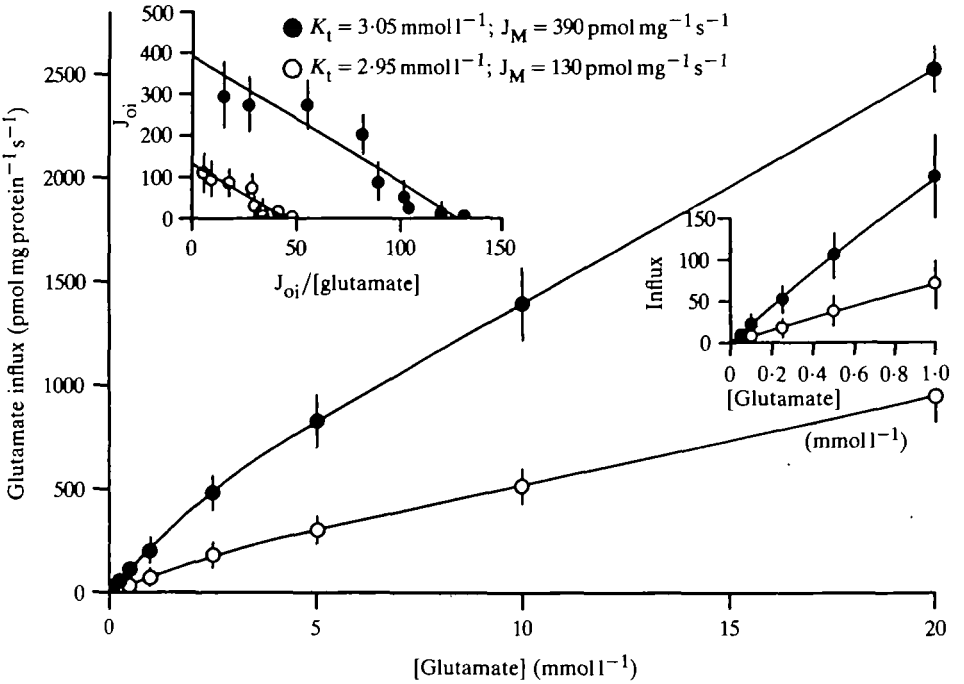

Fig. 7. Effects of external glutamate concentration on L-[^{3}H]glutamate influx (10-s uptake) into lobster brush border membrane vesicles. Vesicles were loaded with 200 mmol l^{-1} mannitol at either pH 4·0 (●) or 6·0 (○) and were incubated in media of the same pH containing 100 mmol l^{-1} NaCl and a selected amino acid concentration ranging from 0·05 to 20 mmol l^{-1}. The inset to the right is an expansion of the influx data obtained for 0·05–1·0 mmol l^{-1} glutamate concentrations. The inset to the left is an Eadie–Hofstee plot of calculated carrier-mediated L-[^{3}H]glutamate influx after subtraction of the apparent diffusion component of amino acid uptake from total transfer shown in the main body of the figure. Kinetic constants displayed on the graph were derived from the slope (K_t) and vertical intercept (J_M) of the Eadie–Hofstee plot using linear regression analysis. Bars indicate ±S.E.M.

permeability' constant. Non-saturable L-[^{3}H]glutamate influx was subtracted from total influx at each amino acid concentration yielding an estimate of carrier transport. Calculated carrier-mediated L-[^{3}H]glutamate influxes were drawn in an Eadie–Hofstee plot (Fig. 7, left-hand inset) to provide estimates of the transport constants K_t and J_M.

The data in Fig. 7 show that pH had a significant effect on the influx of L-[^{3}H]glutamate across hepatopancreatic BBMV. The major impact of a drop in pH on amino acid entry was to triple both the maximal carrier-mediated transport component (J_M at pH 4·0 = 390 ± 72; J_M at pH 6·0 = 130 ± 47 pmol mg^{-1} s^{-1}) and the apparent diffusional permeability of the membrane to this compound (P at pH 4·0 = 111 ± 26; P at pH 6·0 = 42 ± 13 pmol mg^{-1} s^{-1} mmol l^{-1}). The apparent half-saturation constant for amino acid association with transport proteins in the membrane was not significantly ($P > 0·05$) influenced by a reduction in pH (K_t at pH 4·0 = 3·05 ± 0·43; K_t at pH 6·0 = 2·95 ± 0·27 mmol l^{-1}).

Trans-stimulation of L-[^{3}H]glutamate influx by preloaded amino acids

A series of trans-stimulation experiments was performed to ascertain the relative substrate specificity of the glutamate carrier process. Vesicles were preincubated for 90 min on ice prior to influx experiments with 200 mmol l^{-1} mannitol at pH 4·0 and one of 12 different amino acids or amines at 0·5 mmol l^{-1}. Following the loading process, a 10-s influx of 0·05 mmol l^{-1} L-[^{3}H]glutamate from an external medium containing 100 mmol l^{-1} NaCl at pH 4·0 was measured. Longer preloading periods did not significantly alter L-glutamate influx values, suggesting that by 90 min preloading was complete. Fig. 8 indicates that vesicles preloaded with L-aspartate, L-cysteate, D-aspartate, L-glutamate, L-tyrosine, L-asparagine and D-glutamate significantly ($P < 0·05$) stimulated L-[^{3}H]glutamate influx compared to vesicles containing mannitol (control). In contrast, the rate of L-[^{3}H]glutamate influx into vesicles preloaded with 0·5 mmol l^{-1} L-leucine, L-glutamine, L-proline, L-alanine and L-lysine

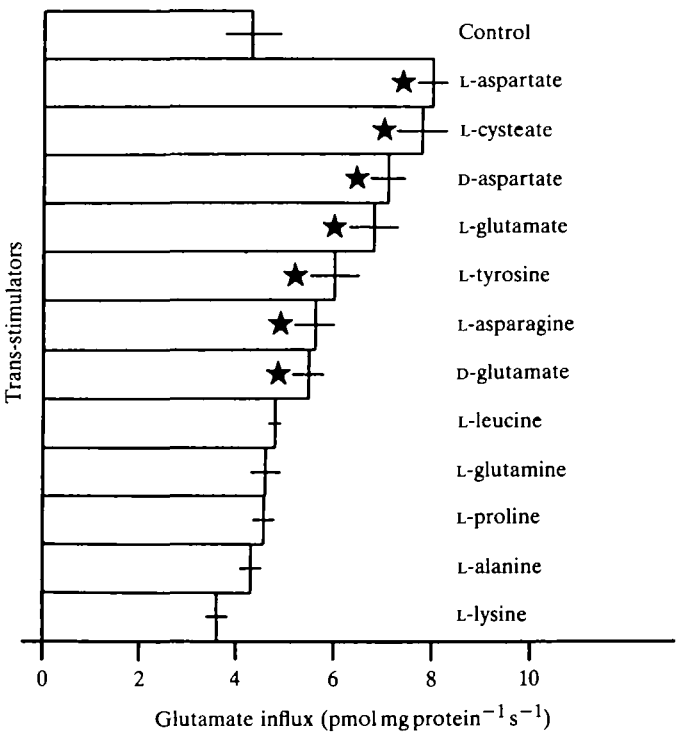

Fig. 8. Effects of preloaded amino acids on influx of 0·05 mmol l^{-1} L-[^{3}H]glutamate in lobster brush border membrane vesicles. Vesicles were preincubated for 90 min on ice prior to experimentation with 200 mmol l^{-1} mannitol at pH 4·0 and one of 12 different amino acids or amines at 0·5 mmol l^{-1}. Following preloading, 10-s uptake of 0·05 mmol l^{-1} L-[^{3}H]glutamate from an external medium containing 100 mmol l^{-1} NaCl at pH 4·0 was measured. The extent of each bar represents the mean of five replicates per treatment, the horizontal lines signify the standard errors, and the stars represent those results that are significantly ($P < 0·05$) different from the control, which was loaded with 0·5 mmol l^{-1} mannitol instead of a test amino acid.

was not significantly ($P > 0{\cdot}05$) different from the rate of entry under control conditions. These results suggest, in general, that amino compounds that structurally resemble L-[^{3}H]glutamate at acidic pH tend to trans-stimulate the uptake of the labelled substrate, probably as a result of common usage of a membrane carrier mechanism. The lack of significant trans-stimulation by the second group of substrates may reflect the independence of these amino compounds from the L-glutamate transport process. These data further indicate that substrate charge and the presence of specific side groups appeared to be more important in common carrier usage than the stereospecific arrangement of the compound, since both D- and L- configurations of otherwise similar amino acids were stimulatory.

Stoichiometric relationship between Na^+, Cl^- and glutamate cotransport

Figs 1 and 3 indicate that L-[^{3}H]glutamate uptake shows a strong dependence upon both external Na^+ and Cl^- which was not satisfied by other cations or anions. To evaluate the nature of these specific ion dependencies, in one experiment L-[^{3}H]glutamate influx at fixed amino acid and Cl^- concentrations was measured as a function of variable external sodium concentration. In a second experiment, L-[^{3}H]glutamate influx was determined at fixed amino acid and Na^+ concentrations using variable concentrations of external chloride. In the first experiment vesicles were loaded with 800 mmol l^{-1} mannitol at pH 4·0 and were incubated for 10 s in media of the same pH containing a fixed Cl^- concentration (400 mmol l^{-1}), 0·05 mmol l^{-1} L-[^{3}H]glutamate, and variable concentrations of Na^+ (TMA^+ replacing Na^+) from 0 to 400 mmol l^{-1}. In the second experiment conditions were the same except that a fixed Na^+ concentration (400 mmol l^{-1}) and variable concentrations of Cl^- (NO_3^- replacing Cl^-) from 0 to 400 mmol l^{-1} were used.

Fig. 9 indicates that amino acid influx was a hyperbolic function of both external Na^+ or Cl^- concentrations at fixed concentrations of the respective counterion. Amino acid influx approached an asymptote at the highest Na^+ or Cl^- concentrations used and displayed significant Na^+- and Cl^--independent transport in the absence of either ion. The Eadie–Hofstee plot (inset) shows the half-saturation constants for apparent Na^+ and Cl^- binding to the amino acid carrier ($K_{Na} = 215 \pm 17$ mmol l^{-1}; $K_{Cl} = 188 \pm 14$ mmol l^{-1}), the maximal Na^+-stimulated L-[^{3}H]glutamate influx ($J_M = 7{\cdot}1 \pm 0{\cdot}6$ pmol mg protein^{-1} s^{-1}), and the maximal Cl^--stimulated L-[^{3}H]glutamate influx ($J_M = 6{\cdot}0 \pm 0{\cdot}6$ pmol mg protein^{-1} s^{-1}). Neither apparent half-saturation constants nor maximal transport velocities were significantly different from one another ($P > 0{\cdot}05$). These data suggest that the Na^+-dependence of L-[^{3}H]glutamate uptake shown in Fig. 1 and the Cl^--dependence of amino acid transfer in Fig. 3 are probably due to cotransport of the cation, anion and amino acid on a shared carrier process. Furthermore, because of the hyperbolic nature of the relationships between variable external ion concentrations and L-[^{3}H]glutamate influx, the stoichiometry of coupled entry is probably 1 Na^+ : 1 Cl^- : 1 glutamate (Turner, 1983).

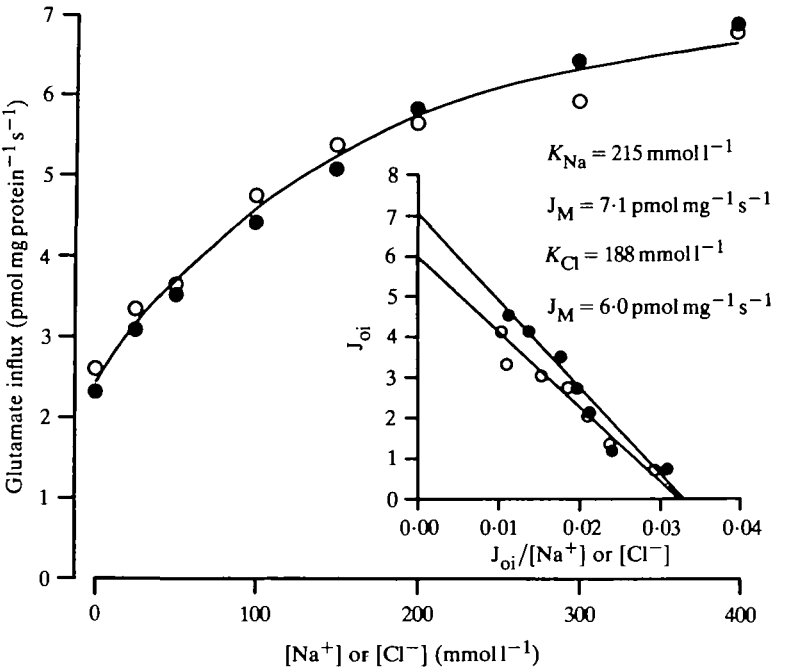

Fig. 9. Effects of variable external Na^+ (●) or Cl^- (○) concentrations on carrier-mediated L-[^{3}H]glutamate influx. When the effect of variable external Na^+ was examined, vesicles were loaded with 800 mmol l^{-1} mannitol at pH 4·0 and incubated for 10 s in media of the same pH containing a fixed Cl^- concentration (400 mmol l^{-1}), 0·05 mmol l^{-1} L-[^{3}H]glutamate and variable concentrations of Na^+ (TMA^+ replacing Na^+) from 0 to 400 mmol l^{-1}. When the effect of variable external [Cl^-] was measured, conditions were the same except that a fixed Na^+ concentration (400 mmol l^{-1}) and variable concentrations of Cl^- (NO_3^{2-} replacing Cl^-) from 0 to 400 mmol l^{-1} were used. The inset shows Eadie–Hofstee plots of the data obtained from the main body of the figure after subtraction of the respective influxes occurring at 0 mmol l^{-1} concentrations of external Na^+ or Cl^-. Lines drawn in this inset were calculated from linear regression analysis and the kinetic constants were obtained from their slopes (K_t) and vertical intercepts (J_M).

DISCUSSION

Nutrient absorption by the crustacean hepatopancreas

In crustaceans, the epithelia-lined tubular system of the hepatopancreas appears to perform a number of physiological activities that are associated with several separate organ systems in the vertebrates, including absorption of nutrients (intestine), synthesis and secretion of digestive enzymes (pancreas), storage of carbohydrate and lipid reserves (liver, fat) and temporary deposition of skeletal calcium (bone). Over the last century investigators have increasingly ascribed a more significant role to the hepatopancreatic tubular system than to the intestine for overall nutrient absorption (Gibson & Barker, 1979; Dall & Moriarty, 1983). Prior to the application of membrane vesicle techniques to invertebrate tissues, however, there had been no direct measurements of organic solute transport into or across hepatopancreatic

epithelial membranes or cells and, therefore, the absorptive role of this structure has remained uncertain.

Several recent studies using purified brush border membrane vesicle preparations of lobster hepatopancreatic epithelial cells have suggested that specific carrier mechanisms for different nutrient groups are found in this location and that their transport properties in some regards are similar to those described for the same substances in vertebrate gut or kidney. In other ways these transporters illustrate unique properties that appear to be related to the physiological environment of the hepatopancreatic tubular system. The major chemical difference between vertebrate and crustacean absorption sites is luminal pH. Whereas luminal pH of vertebrate small intestine is near neutrality, that of hepatopancreatic tubules may vary from pH 4·0 to 6·0. These chemical differences appear to affect both membrane carrier kinetics as well as substrate charge properties in the two animal groups.

In crustaceans, hepatopancreatic Na^+-dependent glucose transport across the brush border membrane is stimulated by a drop in pH as a result of lowering the Michaelis constant for sugar binding and increasing the apparent membrane permeability to the hexose (Ahearn *et al.* 1985). In mammals, an increase in luminal proton concentration strongly inhibits transmembrane sugar transfer due to decreased apparent sugar binding affinity (Toggenburger *et al.* 1978). Alanine and lysine transport in lobster epithelial BBMV are both totally Na^+-independent at biologically relevant acidic pH and cross-inhibit and trans-stimulate one another, suggesting the use of a common carrier mechanism (Ahearn *et al.* 1986; Ahearn & Clay, 1987). In mammals, a significant fraction of alanine transport, and some lysine transport, occur by way of Na^+-dependent membrane proteins (Christensen, Liang & Archer, 1967; Stevens *et al.* 1982). As with vertebrate epithelia, the driving forces for glucose transport in crustaceans appear to be both the transmembrane sodium gradient and the membrane potential (Ahearn *et al.* 1985). In contrast, because of their protonation and conversion to cationic species at acidic luminal pH, the electrical potential difference across the brush border alone appears adequate to facilitate the uptake of alanine and lysine from the crustacean tubular lumen (Ahearn *et al.* 1986; Ahearn & Clay, 1987).

Transport properties of L-[^{3}H]*glutamate in mammalian and crustacean epithelia*

In mammalian intestine and kidney epithelial brush border vesicles, the acidic amino acids are transported by a Na^+-dependent carrier process that employs the combined driving forces of an inwardly directed Na^+ gradient and an outwardly directed K^+ gradient to accumulate the amino acid intravesicularly to considerably higher concentrations than occur extravesicularly (Buckhardt *et al.* 1980; Schneider & Sacktor, 1980; Schneider *et al.* 1980; Corcelli & Storelli, 1983). In addition, membrane potential may provide another driving force for the vesicular uptake of acidic amino acids since $Na^+ + K^+$-dependent glutamate influx is strongly stimulated by an inside negative electrical potential difference (Burckhardt *et al.* 1980). These authors proposed a transport model involving the symport of 3 Na^+ + 1 glutamate in conjunction with the simultaneous antiport of 1 K^+ to account for these observations

(Burckhardt *et al.* 1980). Chloride ion was found to activate specifically the Na^+-dependent acidic amino acid transport system in either the presence or the absence of internal K^+, but the mechanism of this anion effect was unclear (Corcelli & Storelli, 1983).

The present investigation with lobster brush border membrane vesicles indicates that Na^+-dependent L-glutamate cotransport by an apparently specific 'acidic' amino acid transport protein also occurs in these invertebrates. Maximal L-glutamate transport in this preparation was observed at pH 4·0, a value where considerable protonation of the amino acid side group having a pK of 4·3 would be expected, producing a zwitterionic solute (Fig. 2). Therefore, while L-glutamate is transported in mammals as an acidic amino acid, carrying a net negative charge, it is largely electrically neutral in crustaceans. Still, the substrate specificity of the L-glutamate transporter in these invertebrates appears to be similar (Fig. 8) to that displayed by vertebrate intestine or kidney (Schneider *et al.* 1980; Corcelli *et al.* 1982), and a similar membrane protein may facilitate the transfer of the amino acid in both animal groups.

In contrast to the situation shown for mammalian epithelia, Na^+-dependent L-glutamate transport in crustaceans appears to be independent of internal K^+ (Fig. 4). However, Na^+-dependent amino acid transfer in these invertebrates requires the presence of an inwardly directed Cl^- gradient in conjunction with a simultaneous inwardly directed Na^+ gradient (Figs 1, 3). The combined requirements for both Na^+ and Cl^- are due to the simultaneous cotransport of the two ions with the amino acid (Fig. 9). Turner (1983) has suggested that hyperbolic kinetics such as that displayed in Fig. 9 indicates the probable transport stoichiometry of 1 : 1 between the substrates involved. Since variation in both $[Na^+]$ and $[Cl^-]$ independently led to hyperbolic L-glutamate influx kinetics, a transport stoichiometry of $1\,Na^+ : 1\,Cl^- : 1$ glutamate seems likely. Under these conditions, at pH 4·0 where L-glutamate is largely a zwitterion, the cotransport of $1\,Na^+$ and $1\,Cl^-$ with a single zwitterionic amino acid should be electroneutral and unresponsive to changes in membrane potential. Fig. 5 indicates that NaCl-stimulated L-glutamate uptake was unaffected by short-circuiting membrane vesicles using the protonophore CCCP, whereas under these conditions electrogenic Na^+-dependent glucose transfer was markedly reduced as a result of abolishing the electrical coupling between Cl^- diffusion and Na^+-glucose symport. These results provide strong supportive evidence for electroneutral Na^+-Cl^--glutamate cotransport at acidic pH in crustacean hepatopancreatic brush border membrane vesicles.

The absence of both an outwardly directed K^+ gradient and the membrane potential as driving forces for L-glutamate accumulation in crustaceans may neither be deleterious nor render the efficiency of amino acid transport less in these invertebrates than that occurring in mammalian epithelia. Although intracellular Na^+ and Cl^- activities in lobster hepatopancreatic epithelial cells have not been measured, values for intestinal epithelial cells from a marine mollusc, *Aplysia*, have been determined using ion-selective microelectrodes and ranged between 10 and

20 mmol l^{-1} (Gerencser, 1985). Sea water and hepatopancreatic luminal concentrations of Na^+ and Cl^- are approximately 450–500 mmol l^{-1}. If intracellular ion activities in crustaceans and molluscs are similar, approximately 25- to 50-fold concentration gradients for both ions probably occur across the hepatopancreatic epithelial brush border membrane, providing sufficient combined driving force for efficient accumulation of L-glutamate. In fact, maximal L-[^{3}H]glutamate influx at pH 4·0 (390 pmol mg^{-1} s^{-1}; Fig. 7) is approximately 11 times greater than J_M for Na^+-independent, membrane-potential-sensitive L-[^{3}H]alanine influx in the same preparation at the same pH (36 pmol mg^{-1} s^{-1}; Ahearn *et al.* 1986), and is 25 times larger than maximal Na^+-dependent, electrogenic D-[^{3}H]glucose influx at pH 6·0 (14·5 pmol mg^{-1} s^{-1}; Ahearn *et al.* 1985). Furthermore, maximal L-[^{3}H]glutamate influx in lobster BBMV at pH 4·0 is approximately nine times greater than maximal Na^+-dependent glutamate transport in rat intestine (45 pmol mg^{-1} s^{-1}; Corcelli *et al.* 1982).

The evidence presented in this investigation therefore suggests that L-[^{3}H]glutamate transport in crustacean hepatopancreatic brush border membrane occurs by the same carrier mechanism, or at least one with several similar properties, as that described for acidic amino acids in vertebrates. The major functional differences between the animal groups with respect to glutamate transport appear to be related to the cotransported ion ligands. In vertebrates, a combination of Na^+ symport and K^+ antiport occurs by a process that may be electrogenic with the amino acid, and gradients of these respective ions and the membrane potential provide the driving force for glutamate accumulation. In crustaceans, Na^+-Cl^--glutamate symport occurs in the absence of K^+ antiport and the combined inwardly directed ion gradients appear to provide a more than adequate driving force for electroneutral amino acid accumulation in these invertebrates to match that displayed by vertebrate epithelia.

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