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ELECTRONEUTRAL Na^+-2 Cl^--LEUCINE COTRANSPORT BY LOBSTER HEPATOPANCREATIC BRUSH-BORDER MEMBRANE VESICLES

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Summary

Uptake of L-[^{3}H]leucine by lobster hepatopancreatic brush-border membrane vesicles was stimulated by a transmembrane NaCl gradient (o > i), but not by identical gradients of NaSCN or other Cl^- salts (e.g. K^+, Li^+, NH_4^+, Cs^+ or choline), suggesting that amino acid transfer depended upon both Na^+ and Cl^-. In NaCl medium at acidic pH, leucine uptake was largely electroneutral and unresponsive to a transmembrane potential generated by permeable anions; however, in Na^+-free medium, amino acid transport was strongly electrogenic under the same conditions. Leucine influx occurred by a combination of two carrier processes at physiologically acidic pH. One exhibited an influx K_t of 0·59 mmol l^{-1}, a J_M of 390 pmol mg protein^{-1} s^{-1} and a cotransport stoichiometry of 1 Na^+:2 Cl^+:1 leucine. This process was most strongly cis-inhibited by the non-polar amino acids phenylalanine, methionine and isoleucine, and most weakly inhibited by the more polar species methylaminoisobutyric acid (MeAIB), hydroxyproline, glutamate and arginine. The second leucine carrier system showed a very low binding affinity and could not be distinguished from diffusion, was Na^+- and Cl^--independent, and was cis-inhibited by more polar amino acids such as lysine, hydroxyproline, MeAIB, alanine and glutamate. These results suggest that brush-border leucine transport in lobster hepatopancreas at acidic pH may occur by a combination of a modified L-system, that includes ion cosubstrates, and either by a second undefined Na^+-independent process with a broad structural specificity or by multiple Na^+-independent processes.

Introduction

During the last two decades several studies have been conducted on the crustacean gastrointestinal tract to determine the site and properties of nutrient transport in this group of animals. These studies have shown that both the tubular

Key words: cotransport, leucine transport, brush-border membrane vesicles, Na^+-dependent, hepatopancreas, *Homarus americanus*, ion gradients.

intestine and the main gut diverticulum, the hepatopancreas, are organs of sugar, amino acid and vitamin absorption. A series of studies during the 1970s, using isolated intestinal sheets or perfused intestinal preparations from marine and freshwater shrimps, disclosed the presence of high-affinity, low-velocity epithelial brush-border carrier systems for each nutrient class (Ahearn, 1974, 1976, 1982; Ahearn & Maginniss, 1977; Brick & Ahearn, 1978; Maginniss, 1977; Wyban, Ahearn & Maginniss, 1980). However, these investigations also reported reduced or negligible rates of transepithelial transport of both sugars and amino acids in the same preparations (Maginniss, 1977; Ahearn & Maginniss, 1977; Wyban *et al.* 1980), suggesting that the crustacean intestine may not be a significant site for lumen to blood nutrient transfer. Recently, these findings and conclusions have been independently confirmed in a study of intestinal glucose transport in a marine species of crab (Chu, 1986).

The crustacean hepatopancreatic epithelium has been ascribed a nutrient absorptive function for most of this century (Yonge, 1924; van Weel, 1955; Loizzi, 1971; Gibson & Barker, 1979; Dall & Moriarty, 1983). Application of membrane vesicle techniques to this tissue has resulted in the production of purified brush-border membrane preparations that reproducibly transport a variety of sugars and amino acids by carrier processes with kinetic constants quantitatively more closely resembling those of mammalian intestine and kidney than those of crustacean intestine. Lobster hepatopancreatic brush-border membrane vesicles exhibit Na^+-dependent, carrier-mediated glucose transport which is electrogenic and responsive to pH (Ahearn, Grover & Dunn, 1985). Both alanine and lysine transport by these vesicles are Na^+-independent, carrier-mediated and strongly sensitive to transmembrane electrical potential after protonation at acidic pH (Ahearn *et al.* 1986; Ahearn & Clay, 1987*a*). Brush border glutamate transport by these membrane systems at physiologically acidic pH occurs by cotransport with both Na^+ and Cl^- by an electroneutral carrier process (Ahearn & Clay, 1987*b*).

The present study examines the characteristics of leucine transport by lobster hepatopancreatic brush-border membrane vesicles with particular emphasis upon the roles of cations and anions in stimulating the transmembrane transfer of this amino acid. Findings indicate that, at acidic pH, leucine is transferred across the apical membrane by both Na^+-dependent and Na^+-independent carrier processes, the former exhibiting a $1\,Na^+$: $2\,Cl^-$: 1 leucine transport stoichiometry and the latter occurring in the absence of both ions.

Materials and methods

Live intermoult Atlantic lobsters (*Homarus americanus*; 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*a*,*b*). Purification of a final membrane sample was assessed by comparing enzyme activities of this pellet with those of the original tissue homogenate. These 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}H]leucine (ICN Radiochemicals). After incubation (15 s, 1, 2, 5, 10, 20, 120 min), 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 pore diameter), 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 times 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.

Leucine uptake values were expressed as pmol (using specific activity of L-[^{3}H]leucine 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 %. Throughout this study, mean values are given with their standard errors.

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

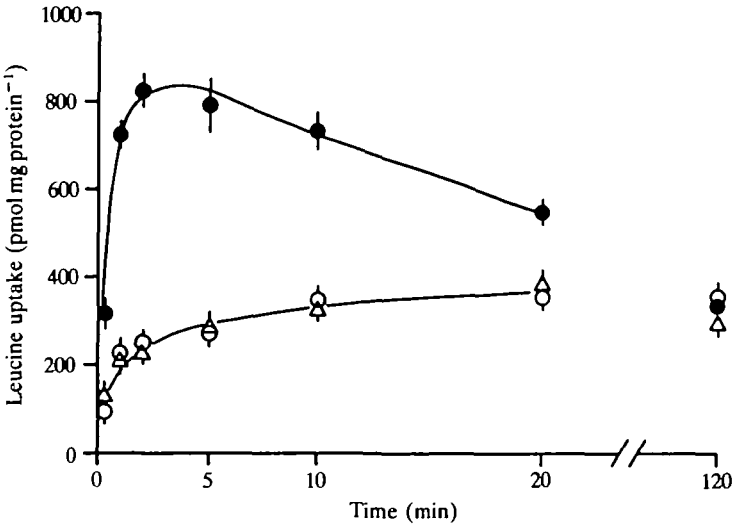

Fig. 1. Sodium dependency of uptake of 0·05 mmol l^{-1} L-[^{3}H]leucine by lobster brush-border membrane vesicles (BBMV). Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 (20 mmol l^{-1} Mes-Tris) and incubated in media at the same pH containing 100 mmol l^{-1} NaCl (●), 100 mmol l^{-1} choline chloride (△) or 200 mmol l^{-1} mannitol (○). All vesicles were short-circuited with 50 μmol l^{-1} CCCP. Vertical lines are ± S.E.M., $N = 3$–5.

Results

Effects of inwardly directed ion gradients on leucine uptake

The effects of inwardly directed cation gradients on the time course of uptake of 0·05 mmol l^{-1} L-[^{3}H]leucine by lobster BBMV were examined by loading the vesicles with 200 mmol l^{-1} mannitol at pH 4·0 and incubating them in media at the same pH containing the labelled amino acid and 100 mmol l^{-1} NaCl, 100 mmol l^{-1} choline chloride or 200 mmol l^{-1} mannitol. The ionophore, CCCP, was present at a concentration of 50 μmol l^{-1} on both vesicle surfaces to short-circuit the preparation and eliminate electrical coupling between transported species. As illustrated in Fig. 1, vesicles incubated in NaCl exhibited significantly greater apparent amino acid influx rates (15 s uptake) than those exposed to the other two media. In addition, a marked leucine uptake overshoot occurred in NaCl (intravesicular amino acid concentration transiently exceeded that at equilibrium, 120 min, by almost a factor of three), but not in the other two media. These results suggest that a transmembrane NaCl gradient is required for concentrative uptake of L-[^{3}H]leucine at pH 4·0.

Fig. 2 shows the effects of various external cations and anions on the apparent influx of 0·05 mmol l^{-1} L-[^{3}H]leucine (10 s uptake) in short-circuited lobster BBMV. Vesicles were loaded with 200 mmol l^{-1} mannitol and 50 μmol l^{-1} CCCP at pH 4·0 and were incubated in media of the same pH and ionophore concentration and either 200 mmol l^{-1} mannitol or 100 mmol l^{-1} NaCl, KCl, LiCl, NH_4Cl,

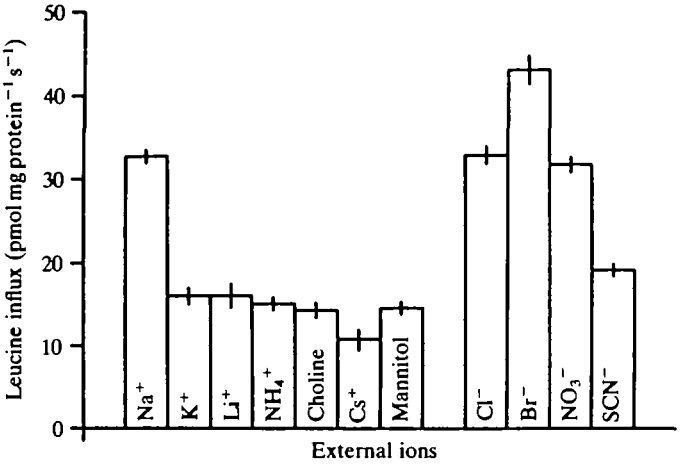

Fig. 2. Effects of inwardly directly cation and anion gradients on apparent influx of 0·05 mmol l^{-1} L-[^{3}H]leucine into lobster BBMV. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 (20 mmol l^{-1} Mes-Tris) and incubated for 10 s in media of the same pH containing 100 mmol l^{-1} of a Cl^- salt, 100 mmol l^{-1} of a Na^+ salt or 200 mmol l^{-1} mannitol. All vesicles were short-circuited with 50 μmol l^{-1} CCCP. Vertical lines are ± S.E.M., $N = 3$–5.

choline chloride, CsCl, NaBr, $NaNO_3$ or NaSCN. This figure indicates that apparent leucine influx exhibited a restricted cation specificity, accepting only Na^+ as a stimulatory species, but a somewhat broad anion specificity, being strongly enhanced by Cl^-, Br^- and NO_3^-, but only weakly by SCN^-. Because all vesicles were short-circuited in these experiments, the stimulatory effects of anions cannot be attributed to electrical coupling between diffusional anion flow and Na^+-leucine cotransport, but rather suggest the association of both Na^+ and an anion with the leucine transporter during the translocation process.

Sensitivity of leucine uptake to membrane potential

Our previous work has suggested that transport of D-[^{3}H]glucose 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; Ahearn & Clay, 1987*b*). The concurrent presence of an inwardly directed gradient of a permeable anion stimulated Na^+-dependent glucose uptake owing to electrical coupling between the transported solutes. This electrical coupling was abolished in short-circuited vesicles (CCCP) and Na^+-dependent glucose uptake was markedly reduced.

To characterize the sensitivity of leucine transport to membrane potential, the effects of CCCP on leucine uptake in the presence and absence of an inwardly directed NaCl gradient at pH 5·0 were compared with the effects of this ionophore on Na^+-dependent glucose transport in the same preparation. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 5·0 and 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]leucine. A third group of vesicles served as the control for leucine uptake and was loaded similarly, but was incubated in a medium containing 100 mmol l^{-1} choline chloride at the same pH. A portion of each group of vesicles was preincubated in 50 μmol l^{-1} CCCP for 30 min prior to experimentation.

Fig. 3 shows that, in the absence of the ionophore, glucose uptake exhibited a strong NaCl dependency which was abolished when the vesicles were short-circuited. In contrast, the ionophore had only a small, but significant, effect on leucine uptake in NaCl medium, suggesting that NaCl-dependent amino acid transport was largely electroneutral and unresponsive to membrane potential, whereas Na^+-dependent glucose uptake was strongly electrogenic.

In choline chloride medium, leucine uptake was considerably slower than in NaCl, as previously shown in Fig. 1, and was strongly reduced by the presence of the ionophore. These results are consistent with an electrogenic stimulation of leucine transport in the absence of Na^+ by a transmembrane Cl^--generated diffusion potential. They suggest that, at acid pH, leucine was transferred across the membrane as a protonated cation responsive to an electrically negative vesicular interior. In NaCl medium this electrogenic process apparently makes a minor contribution to total amino acid accumulation.

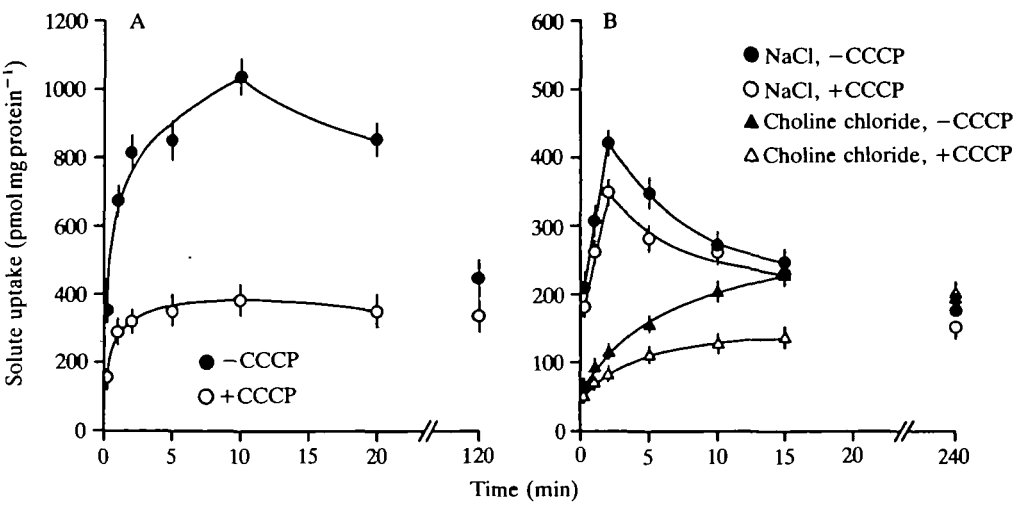

Fig. 3. Effects of the ionophore, CCCP, on the time course of uptake of 0·05 mmol l^{-1} D-[^{3}H]glucose (A) and L-[^{3}H]leucine (B) by lobster BBMV. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 5·0 (12 mmol l^{-1} Mes-Tris) and those exposed to glucose were incubated in media of the same pH containing 100 mmol l^{-1} NaCl, whereas those incubated with leucine were exposed to either 100 mmol l^{-1} NaCl (circles) or 100 mmol l^{-1} choline chloride (triangles). Some of each group of vesicles were preincubated for 30 min in 50 μmol l^{-1} CCCP (filled symbols) before the experiment. Vertical lines are ± S.E.M., $N = 3$–5.

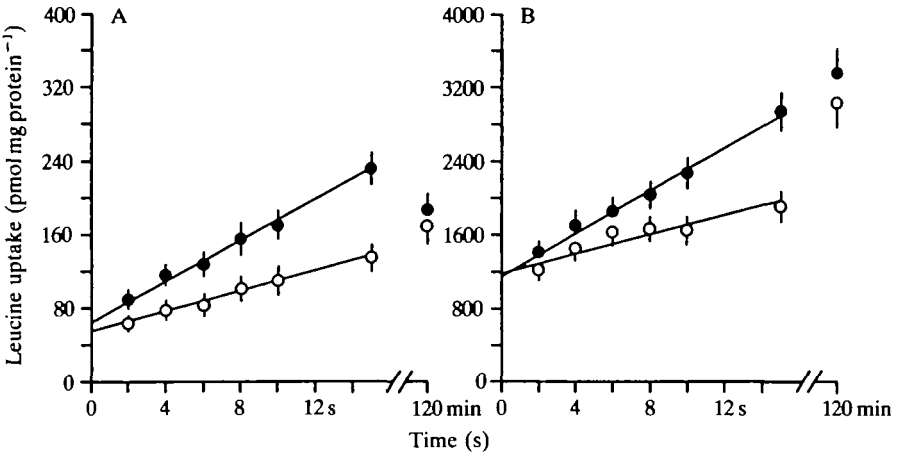

Fig. 4. Effects of external cations on the time course of uptake of 0·05 mmol l^{-1} (A) and 1·0 mmol l^{-1} (B) L-[^{3}H]leucine over very short intervals (2, 4, 6, 8, 10, 15 s and 120 min) by lobster BBMV. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 (20 mmol l^{-1} Mes-Tris) and incubated in media of the same pH containing 100 mmol l^{-1} NaCl (●) or 100 mmol l^{-1} KCl (○) and the radiolabelled amino acid. All vesicles were short-circuited with 50 μmol l^{-1} CCCP. Lines were drawn by linear regression analysis. Vertical bars are ± S.E.M., $N = 3$–5.

Kinetics of leucine influx

The time course of uptake of 0·05, 1·0 and 20·0 mmol l^{-1} L-[^{3}H]leucine by lobster BBMV was examined at very short intervals (2, 4, 6, 8, 10, 15 s and equilibrium at 120 min) using a rapid uptake apparatus to assess accurately the initial entry rates of the amino acid. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 and incubated in media containing either 100 mmol l^{-1} NaCl or 100 mmol l^{-1} KCl and the respective labelled amino acid concentration at the same pH. All vesicles were short-circuited with 50 μmol l^{-1} CCCP. Uptake of radiolabelled leucine was a linear function of time at 0·05 and 1·0 mmol l^{-1} from 2 to 15 s of incubation in both NaCl and KCl media (Fig. 4) The time course of uptake of 20 mmol l^{-1} L-[^{3}H]leucine was linear for only the first 10 s of incubation (data not shown). Straight lines drawn through the data in Fig. 4 were based on linear regression analysis and provide an estimate of unidirectional influx (slope) and binding (extrapolated vertical intercept) of L-leucine. Binding values in NaCl or KCl media at each amino acid concentration were not significantly different ($P > 0·05$) from one another 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]leucine in NaCl and KCl media were 11·2 ± 2·8 and 5·4 ± 1·3 pmol mg protein^{-1} s^{-1}, respectively, while those of 1·0 mmol l^{-1} L-[^{3}H]leucine in the two media were 115·0 ± 14·3 and 45·0 ± 6·1 pmol mg protein^{-1} s^{-1}, respectively. Significant ($P < 0·01$) binding of L-[^{3}H]leucine to vesicles at 0·05 and 1·0 mmol l^{-1} leucine occurred in both media. This binding averaged

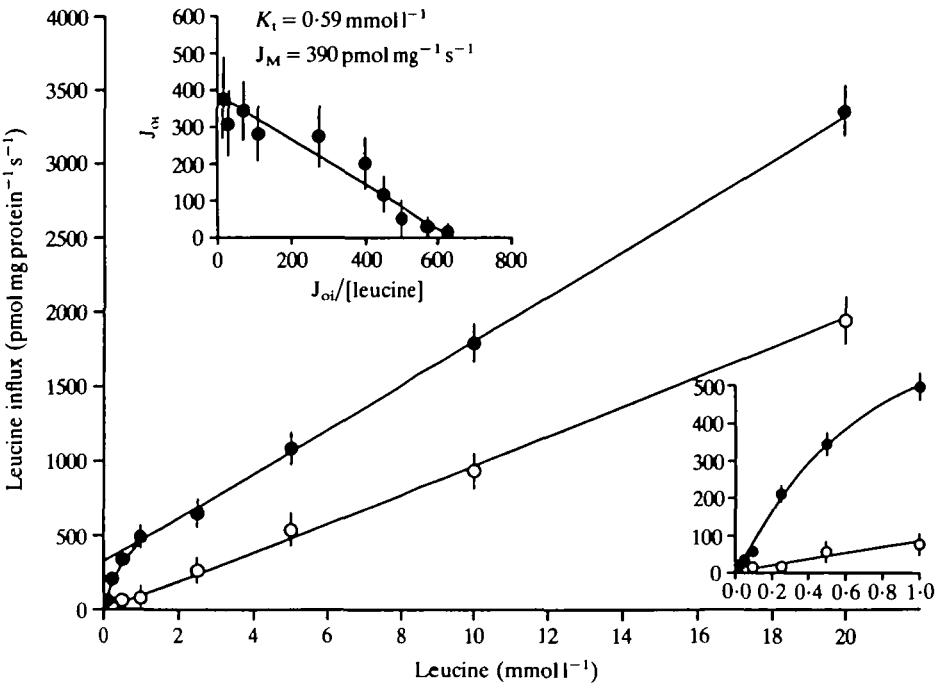

Fig. 5. Effects of external leucine concentration on L-[^{3}H]leucine influx (10-s uptake) into lobster BBMV. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 (20 mmol l^{-1} Mes-Tris) and incubated in media of the same pH containing either 100 mmol l^{-1} NaCl (●) or 100 mmol l^{-1} KCl (○) and amino acid at the selected concentration (0·025–20 mmol l^{-1}). All vesicles were short-circuited with 50 μmol l^{-1} CCCP. The inset on the right is an expansion of the influx data obtained for 0·025–1·0 mmol l^{-1} leucine. The inset on the left is an Eadie–Hofstee plot of calculated carrier-mediated L-[^{3}H]leucine influx after subtraction of bound activity (determined by the method described in Fig. 4) and the linear component of amino acid uptake from total transfer at each amino acid concentration. 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., N = 3–5.

47·5 % and 64·5 % of total uptake during 10-s incubations in NaCl and KCl media, respectively. Similar percentages of total uptake due to binding in the two media occurred for 20·0 mmol l^{-1} L-leucine. These surface binding values were used to correct subsequent 10-s uptake (influx) measurements of this amino acid in NaCl and KCl solutions over a leucine concentration range of 0·05–20 mmol l^{-1}.

Fig. 5 shows the effects of NaCl and KCl on influx of L-[^{3}H]leucine from incubation medium to vesicular interior over a wide amino acid concentration range. The experiment was performed in the manner described in Fig. 4. In all cases vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0, short-circuited with 50 μmol l^{-1} CCCP and incubated for 10 s in media containing the labelled amino acid and either 100 mmol l^{-1} NaCl or 100 mmol l^{-1} KCl at the same pH. In

NaCl medium, leucine influx was a curvilinear function of external amino acid concentration 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 system with a rate that was proportional to the external amino acid concentration. These two processes operating together can be described by the equation:

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

where J_{oi} is the total L-[^{3}H]leucine influx in pmol mg protein^{-1} s^{-1}, J_M is the maximal carrier-mediated influx, K_t is the leucine concentration resulting in half-maximal influx, [L] is the external amino acid concentration and P is the rate constant of the linear process, which may be transmembrane leucine diffusion, carrier transport by a second system exhibiting very low affinity for the amino acid, or a combination of these two. In NaCl medium, non-saturable influx of L-[^{3}H]leucine was subtracted from total influx at each amino acid concentration to yield an estimate of NaCl-dependent carrier transport. Calculated NaCl-dependent carrier-mediated leucine influxes were drawn in an Eadie–Hofstee plot (Fig. 5, lefthand inset) to provide estimates of the transport constants K_t and J_M. In NaCl medium, leucine transport constants were: K_t, 0·59 ± 0·23 mmol l^{-1}, J_M, 390 ± 106 pmol mg protein^{-1} s^{-1}; and P, 147 ± 9·3 pmol mg protein^{-1} s^{-1} mmol l^{-1}. In KCl medium the rate constant of the linear process (P) was 92·5 ± 11·4 pmol mg protein^{-1} s^{-1} (mmol l^{-1})$^{-1}$.

Cis-inhibition of leucine influx in NaCl medium

Several amino acids, known to be transported by a variety of distinct carrier mechanisms in other tissues, were examined for their potential cis-inhibitory effects on apparent leucine influx (10-s exposure uncorrected for binding) in NaCl medium into lobster BBMV. Short-circuited (50 μmol l^{-1} CCCP) vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 and incubated in media of the same pH containing 100 mmol l^{-1} NaCl, 0·05 mmol l^{-1} L-[^{3}H]leucine and 0·5 mmol l^{-1} of the test amino acid. Two control conditions were used: (1) 0·5 mmol l^{-1} mannitol in 100 mmol l^{-1} NaCl; and (2) 0·5 mmol l^{-1} mannitol in 100 mmol l^{-1} KCl. As shown in Fig. 6, control leucine uptake in KCl in the presence of external mannitol was approximately half that in the control condition in NaCl medium, confirming the stimulatory nature of NaCl on apparent leucine influx in the absence of an effective cis-inhibitor.

In external NaCl medium, cis-inhibitory amino acids appeared to fall into three major groups, depending upon the degree of inhibition exerted upon leucine uptake. Group 1 represented the strongest cis-inhibitors, reducing total L-[^{3}H]leucine uptake in NaCl medium by 42–50 %, and included phenylalanine, methionine and isoleucine. Group 2 was moderately inhibitory, lowering apparent L-[^{3}H]leucine influx in NaCl by 20–33 % and consisted of leucine,

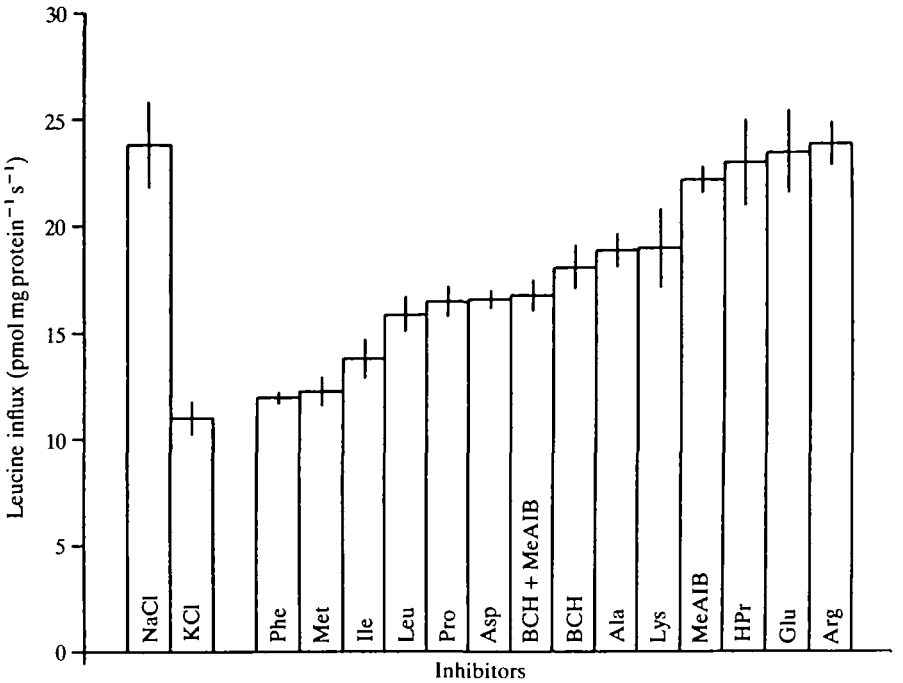

Fig. 6. Effect of cis amino acid inhibitors on 10-s apparent influx of leucine in NaCl medium without correction for bound activity. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 (20 mmol l^{-1} Mes-Tris) and incubated in media of the same pH containing 100 mmol l^{-1} NaCl, 0·05 mmol l^{-1} L-[^{3}H]leucine and 0·5 mmol l^{-1} of cis-inhibitors (0·25 mmol l^{-1} each of BCH + MeAIB). NaCl and KCl controls contained 0·5 mmol l^{-1} mannitol in the external medium. All vesicles were short-circuited with 50 μmol l^{-1} CCCP. Vertical lines were ± S.E.M., $N = 3$–5.

proline, aspartate, BCH [2-aminobicyclo(2,2,2)heptane-2-carboxylic acid], alanine and lysine. Group 3 exhibited virtually no inhibitory effect on L-[^{3}H]leucine uptake and was composed of MeAIB (alpha-methylaminoisobutyric acid), hydroxy-L-proline, glutamate and arginine. These results suggest that leucine uptake in NaCl medium is most strongly inhibited by non-polar amino acids and least affected by polar species.

Nature of Na^+ and Cl^- dependencies of leucine influx

Figs 1 and 2 indicate that uptake of L-[^{3}H]leucine shows a strong dependence upon both external Na^+ and external Cl^- (Br^- and NO_3^- adequately substituting for Cl^-). In addition, Fig. 3 shows that in NaCl medium, uptake of L-[^{3}H]leucine is largely electroneutral and uninfluenced by a transmembrane potential. To evaluate these ion dependencies and the electroneutral nature of leucine transfer, two experiments were conducted. In the first, L-[^{3}H]leucine influx was measured at fixed amino acid and Cl^- concentrations with variable concentrations of Na^+. In

the second, leucine influx was determined at fixed amino acid and Na^+ concentrations, while Cl^- concentrations were varied. In the first experiment, short-circuited 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 400 mmol l^{-1} Cl^{-1}, 0·05 mmol l^{-1} L-[^{3}H]leucine and variable concentrations of Na^+ (choline substituting for Na^+) from 0 to 400 mmol l^{-1}. In the second experiment, conditions were the same except that vesicles were loaded with 1000 mmol l^{-1} mannitol and incubated at a fixed Na^+ concentration (500 mmol l^{-1}) and concentrations of Cl^- (SCN^- replacing Cl^-) from 0 to 500 mmol l^{-1}.

Fig. 7A indicates that influx of leucine was a hyperbolic function of external Na^+ concentration in the presence of a near-saturating Cl^- concentration. Significant ($P < 0·05$) leucine influx occurred in the absence of Na^+ and saturating influx rates were approached at the highest Na^+ concentrations tested. The Eadie–Hofstee plot (inset) shows the half-saturation constant for apparent Na^+ binding to the amino acid carrier ($K_{Na} = 94 \pm 11$ mmol l^{-1}) and the maximal Na^+-stimulated leucine influx ($J_M = 54 \pm 5$ pmol mg protein^{-1} s^{-1}). Because of the hyperbolic

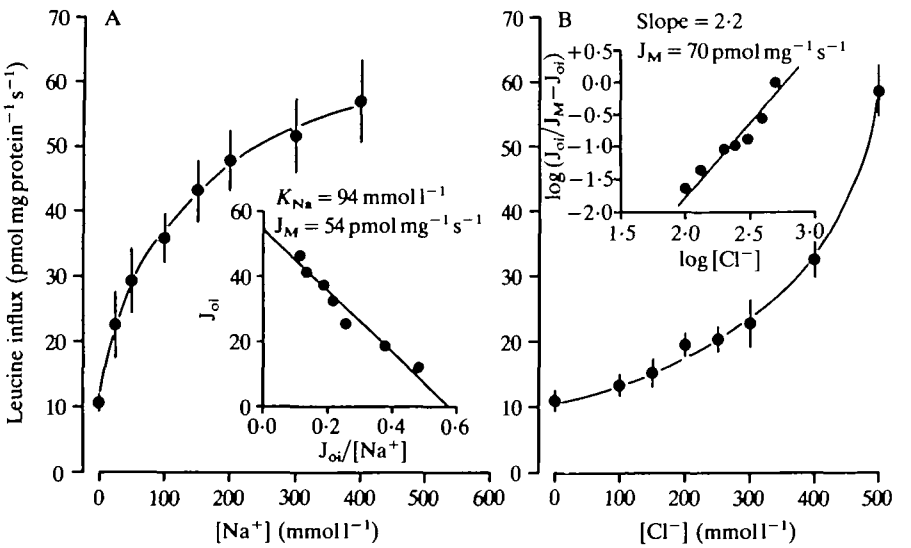

Fig. 7. Effects of variable Na^+ (A) and Cl^- (B) concentrations on carrier-mediated leucine influx (10-s uptake). In the variable [Na^+] experiment, vesicles were loaded with 800 mmol l^{-1} mannitol at pH 4·0 (20 mmol l^{-1} Mes-Tris) and incubated in media of the same pH containing a fixed Cl^{-1} concentration (400 mmol l^{-1}) and variable concentrations of Na^+ (choline replacing Na^+). The inset is an Eadie–Hofstee plot of the results and the kinetic constants were calculated as discussed in Fig. 5. In the variable [Cl^-] experiment, vesicles were loaded with 1000 mmol l^{-1} mannitol at pH 4·0 and incubated in media of the same pH containing a fixed Na^+ concentration (500 mmol l^{-1}) and variable concentrations of Cl^- (SCN^- replacing Cl^-). The inset is a log–log plot of the data assuming Cl^--dependent J_M to be approximately 70 pmol mg protein^{-1} s^{-1}. The slope of the line was calculated by regression analysis. J_{oi} represents leucine influx (pmol mg^{-1} s^{-1}). All vesicles in both experiments were short-circuited with 50 μmol l^{-1} CCCP. Vertical lines are ± S.E.M., $N = 3$–5.

nature of the influx dependency upon [Na^+], a 1 Na^+: 1 leucine stoichiometry can be inferred from these results (Turner, 1983).

Fig. 7B shows that leucine influx was an apparently exponential function of external Cl^- concentration at near saturating concentrations of external Na^+. Significant leucine influx occurred in the absence of external Cl^-, and no indication of saturation was observed at the highest anion concentration used. At an estimated Cl^--dependent J_M of 70 pmol mg protein^{-1} s^{-1}, the slope of the log–log plot, shown as the inset to this figure, was approximately 2·2 ± 0·2, suggesting the occurrence of a 2 Cl^-: 1 leucine influx stoichiometry. The results of these two experiments taken together suggest that the Na^+ and Cl^- dependencies of leucine influx in lobster BBMV are the result of a probable cotransport of these three ligands by a common carrier mechanism with a transport stoichiometry of 1 Na^+: 2 Cl^-: 1 leucine.

Na^+-independent, carrier-mediated leucine uptake

Effect of cis-inhibitors

Figs 1–5 suggest that, in the absence of exogenous Na^+, leucine uptake was strongly depressed in lobster hepatopancreatic BBMV. To determine if leucine uptake by these membrane preparations occurred by carrier mediation in the absence of Na^+, apparent L-[^{3}H]leucine influxes (10-s uptake uncorrected for binding) were measured in choline chloride medium in the presence of a variety of potential cis-inhibitory amino acids. Short-circuited (50 μmol l^{-1} CCCP) vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 and incubated at the same pH in 100 mmol l^{-1} choline chloride containing 0·05 mmol l^{-1} L-[^{3}H]leucine and 0·5 mmol l^{-1} of cis-inhibitors. Three control external concentrations were used at the same pH: (1) 0·5 mmol l^{-1} mannitol in 100 mmol l^{-1} NaCl, (2) 200 mmol l^{-1} mannitol, and (3) 0·5 mmol l^{-1} mannitol in 100 mmol l^{-1} choline chloride.

Fig. 8 shows that apparent leucine influxes in mannitol and choline chloride controls were approximately 50 % of that in NaCl, confirming the stimulatory nature of NaCl for transfer of this amino acid. Fig. 8 also indicates that certain amino acids strongly inhibited leucine uptake by short-circuited vesicles in choline chloride, while others exerted little if any effect. Amino acids which were effective cis-inhibitors of apparent leucine influx in choline chloride were generally polar compounds, and were those that most weakly inhibited leucine entry in NaCl. At the concentrations used, the strongest inhibitors of Na^+-independent leucine uptake were lysine (58 % reduction from the level of the choline chloride control), hydroxyproline, MeAIB, BCH, alanine and glutamate. Phenylalanine was the most effective cis-inhibitor of leucine uptake in NaCl medium, but only caused a 19 % decrease of leucine entry in choline chloride. The non-polar amino acids phenylalanine, leucine and methionine were weak inhibitors of apparent leucine influx in the absence of sodium. These results suggest that leucine is transported by lobster hepatopancreatic BBMV by at least two different carrier processes, one NaCl-dependent and preferring non-polar amino acids as substrates, and the other

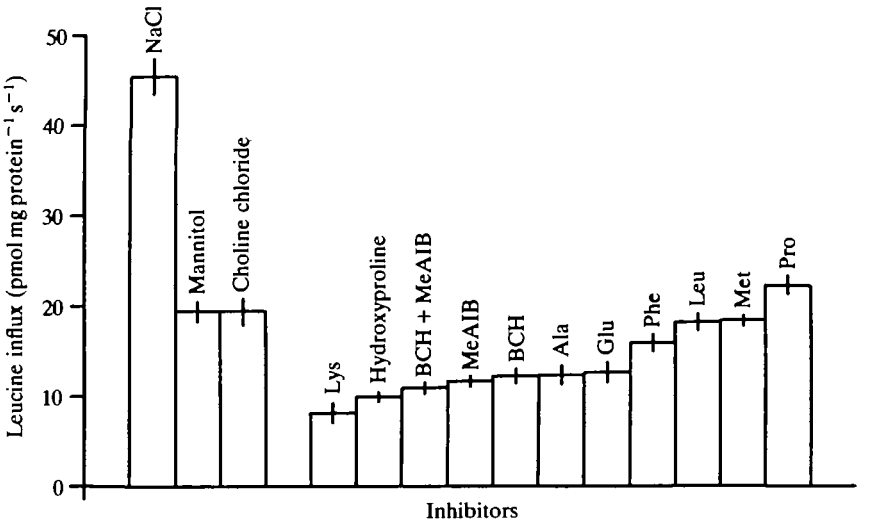

Fig. 8. Effect of cis amino acid inhibitors on 10-s apparent influx of leucine in choline chloride medium without correction for bound activity. Vesicles were loaded with 200 mmol l^{-1} mannitol at pH 4·0 (20 mmol l^{-1} Mes-Tris) and incubated in media of the same pH containing 100 mmol l^{-1} choline chloride, 0·05 mmol l^{-1} L-[^{3}H]leucine and 0·5 mmol l^{-1} of cis-inhibitors (0·25 mmol l^{-1} each of BCH and MeAIB). NaCl and choline chloride controls contained 0·5 mmol l^{-1} mannitol in the external medium. All vesicles were short-circuited with 50 μmol l^{-1} CCCP. Vertical lines are ± S.E.M., $N = 3$–5.

Na^+-independent with a preference for more polar compounds. At present the mechanism of inhibition of leucine transport by any amino acid in NaCl or choline chloride is unclear.

Cl^--independence of the transfer process

Figs 1–5 suggested that in the absence of external Na^+, an inwardly directed 100 mmol l^{-1} Cl^- gradient was unable to stimulate directly the uptake of L-[^{3}H]leucine in short-circuited vesicles. In addition, Fig. 3B indicated that leucine was transported largely as a protonated cation at acidic pH, and that transport was strongly stimulated by a transmembrane potential in Na^+-free medium. Both of these results imply, but do not conclusively prove, the Cl^--independence of carrier-mediated leucine transport in the absence of Na^+.

To verify the Cl^--independence of carrier-mediated leucine uptake in Na^+-free medium, short-circuited (50 μmol l^{-1} CCCP) vesicles were loaded with 1000 mmol l^{-1} mannitol at pH 4·0 and incubated in media containing 0·05 mmol l^{-1} L-[^{3}H]leucine and varying concentrations of choline chloride (mannitol substituting for the salt) at the same pH. Fig. 9 shows that apparent leucine influx (10-s uptake uncorrected for binding) was unaffected by choline chloride at concentrations ranging from 0 to 500 mmol l^{-1}. These results confirm the Cl^--independence of carrier-mediated leucine transport in the absence of sodium.

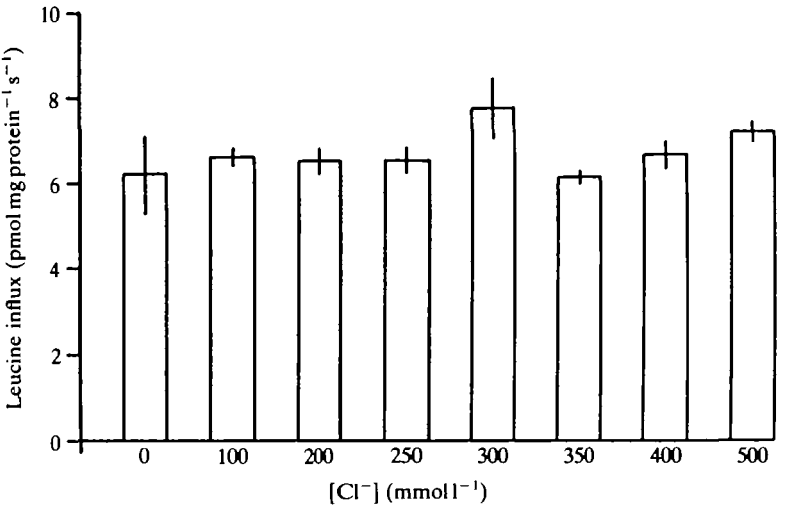

Fig. 9. Effects of variable external Cl^- concentration on Na^+-independent apparent leucine influx without subtraction of bound activity. Vesicles were loaded with 1000 mmol l^{-1} mannitol at pH 4·0 (20 mmol l^{-1} Mes-Tris) and incubated for 10 s in media at the same pH containing 0·05 mmol l^{-1} L-[^{3}H]leucine and choline chloride concentrations ranging from 0 to 500 mmol l^{-1} (mannitol replacing the salt). All vesicles were short-circuited with 50 μmol l^{-1} CCCP. Vertical lines are ± S.E.M., $N = 3$–5.

Discussion

Nutrient transport by the crustacean hepatopancreas at acidic pH

The hepatopancreas is an epithelium-lined tubular diverticulum of the crustacean gut. It performs a number of physiological activities that are associated with several separate organ systems in mammals and other vertebrates, including absorption of nutrients (intestine), synthesis and secretion of digestive enzymes (pancreas, stomach), storage of carbohydrate and lipid reserves (liver, fat) and temporary deposition of skeletal calcium (bone). In recent years, several studies using purified brush-border membrane vesicle preparations of lobster hepatopancreatic epithelial cells have disclosed a variety of Na^+-dependent and Na^+-independent carrier processes for sugars and amino acids (Ahearn *et al.* 1985, 1986; Ahearn & Clay, 1987*a*,*b*). These studies generally found that, because of the *in vivo* acidic nature of the tubular lumen (pH 4·0–6·0), nutrient transport properties of the epithelial brush border were often quite different for a specific solute from those described for the same molecule in mammalian intestine or kidney, where luminal pH is closer to neutrality. Two types of responses to reduced luminal pH were observed in hepatopancreatic vesicle preparations: (1) increased proton concentration significantly altered the kinetic constants of amino acid transport (K_t or J_M) and (2) acidic pH resulted in protonation of charged groups on substrates with appropriate pKa values, chemically changing

the 'electrical signature' of these molecules as perceived by the membrane-bound transport system.

In all the above cases a decrease in pH significantly altered one or both of the transport kinetic constants. Whereas a reduction in luminal pH increased apparent glucose binding affinity (K_t), it was without influence on maximal sugar transport velocity (J_M) (Ahearn *et al.* 1985). In contrast, lowering pH from 6·0 to 4·0 tripled the maximal velocity of glutamate transport, but had no significant effect on apparent glutamate binding (Ahearn & Clay, 1987*b*). Lastly, a mixed effect of pH on alanine transport was observed: a reduction of pH from 6·0 to 4·0 decreased binding affinity, but increased maximal transport velocity by a factor of 10 (Ahearn *et al.* 1986).

Considering the second type of response, alteration of substrate charge, due to protonation of the organic solute itself at low pH, also had marked effects upon nutrient transport. Glucose transport by hepatopancreatic brush-border membrane vesicles was Na^+-dependent, electrogenic and significantly accelerated by a drop in pH (Ahearn *et al.* 1985). Because the glucose molecule remained electrically neutral over a wide pH range, the major factor responsible for increased transport of the sugar at low pH was the increased binding affinity to the transporter. In contrast, alanine transport by the same preparation occurred largely by a Na^+-independent carrier process, but one that responded to a transmembrane potential created by permeable anions or a K^+ diffusion potential in the presence of valinomycin (Ahearn *et al.* 1986). A decrease in pH stimulated Na^+-independent alanine transport by converting increasing amounts of the amino acid into cationic form, which was the charged species preferred by the transporter. Increasing the concentration of cationic alanine at acidic pH resulted in a very marked increase in the maximal rate of carrier-mediated transport. Finally, glutamate influx at acidic pH was characterized by a tripling of maximal carrier transport velocity, in a similar manner to that described for alanine. In this case, however, glutamate was converted to a zwitterionic species at pH 4·0 and was accompanied by one Na^+ and one Cl^- during electroneutral transmembrane transfer (Ahearn & Clay, 1987*b*).

Na^+-dependent and Na^+-independent leucine transport in lobster brush-border membrane vesicles

The present investigation has disclosed two distinct carrier processes for leucine, at acidic pH, in the brush border of hepatopancreatic epithelium. One transport system is shared by both Na^+ and Cl^- and exhibits a transport stoichiometry of $1\,Na^+ : 2\,Cl^- : 1$ leucine. This process is electroneutral and largely inhibited by non-polar amino acids such as phenylalanine and methionine. The second leucine transport system is Na^+- and Cl^--independent and is strongly electrogenic, responding markedly to a transmembrane potential generated by permeable anions. This second carrier process appears to prefer more polar amino acids than the first one.

In a variety of animal cells, neutral amino acids are known to be transported by several distinct carrier processes with discrete substrate requirements (Christensen, 1975; Guidotti, Borghetti & Gassola, 1978; Lerner, 1978; Kilberg, 1982; Mircheff, Kippen, Hirayama & Wright, 1982; Stevens, Ross & Wright, 1982). In addition, the cationic amino acids, such as lysine and arginine, and the acidic amino acids, typified by glutamate and aspartate, are generally transported independently of carriers serving the neutral species (Christensen, 1964; Schultz, Yu-tu, Alvarez & Curran, 1970; Garcia-Sancho, Sanchez & Christensen, 1977; Burckhardt, Kinne, Stange & Murer, 1980; Schneider & Sacktor, 1980; Hammerman, 1982; Steiger, Stange, Biber & Murer, 1983). Neutral amino acids can be transferred across cell membranes by both Na^+-dependent and Na^+-independent carrier systems, often with overlapping specificities. Although there is some disagreement on the number of distinct neutral amino acid transporters, several with somewhat conservative transport properties appear to occur in many cell types from animals of different phyla. Systems A and ASC are both Na^+-dependent and transport neutral amino acids with short, polar or linear side chains. One characterizing substrate for system A is MeAIB. System L is defined as being a Na^+-independent neutral amino acid transporter that prefers substrates with non-polar side chains. A characterizing substrate for system L is BCH. Other identified amino acid transport systems include system Gly (glycine) and system N (histidine, glutamine, etc.) (Kilberg, 1982).

The Na^+-dependence and cis-inhibitor properties disclosed for both hepatopancreatic leucine transporters in the present investigation do not fit easily into the defined characteristics of the neutral amino acid carrier systems discussed above. Neither Na^+-dependent system (A or ASC), as described for other animal cell types, exhibits a Cl^--dependency as shown for hepatopancreatic brush-border membranes (Figs 2, 7). In addition, systems A and ASC exhibit a strong preference for alanine, proline, hydroxyproline and MeAIB, whereas Na^+-independent system L largely transports the non-polar amino acids such as methionine, isoleucine, phenylalanine and BCH (Guidotti *et al.* 1978). The NaCl-dependent leucine transporter in hepatopancreatic membranes clearly preferred non-polar neutral amino acids (Fig. 6), whereas the Na^+-independent hepatopancreatic leucine transporter preferred more polar species (Fig. 8). These results suggest that the NaCl-dependent lobster transporter is an L system that has developed ion dependencies, possibly for energetic considerations as a result of the acidic nature of the absorption site. Christensen (1977) has indicated, in discussing the Ehrlich ascites tumour cell, that if the pH of the extracellular environment is lowered, the selectivity of system L decreases, and amino acids which are taken up exclusively by system A or system X_{AG} (aspartate, glutamate) at neutral pH enter the cell by system L. Although apparent influx of leucine by the NaCl-dependent system is most strongly inhibited by system L amino acids (e.g. phenylalanine, methionine, isoleucine), the moderate reduction in leucine entry in the presence of proline and alanine suggests that loss or reduction of system A (and perhaps system ASC), at the physiologically acidic pH in these

animals, may have occurred. System X_{AG} appears to be largely preserved in these membranes at acidic pH, but cotransports its substrates in zwitterionic form with Na^+ and Cl^- (Ahearn & Clay, 1987*b*).

Assigning a specific carrier system to Na^+-independent leucine transport in lobster membranes is not possible with the available evidence. Although it is clear, as a result of cis-inhibition experiments (Fig. 8), that different systems are operative in the absence and presence of Na^+ the sequence of amino acid effects do not suggest which system may be involved. Lysine was most effective in reducing Na^+-independent leucine influx, whereas the non-polar amino acids (phenylalanine, leucine and methionine) had only minimal inhibitory effects. Although systems A and ASC could be ruled out as transport agents under these acidic conditions because of Na^+-independency, moderate inhibition by MeAIB and alanine (system A), hydroxyproline (system ASC) and BCH (system L) suggest either that Na^+-independent leucine transport at acidic pH occurs by a unique transporter with very broad requirements or that more than one Na^+-independent carrier process for leucine is present in this tissue.

Nature of the driving forces for nutrient transport in hepatopancreatic brush-border vesicles

Sugar and amino acid transport at acidic pH by crustacean hepatopancreatic brush-border membrane vesicles appear to be driven by a combination of a transapical electrical potential and chemical gradients for Na^+ and Cl^-. Although neither the transmembrane potential nor the intracellular Na^+ or Cl^- activities have been measured directly in lobster hepatopancreatic cells, values for these parameters are given in the literature for crustacean and mollusc gastrointestinal epithelial cells, and can be used to provide a basis for establishing the nature of the driving forces for nutrient transport in *Homarus*. Ahearn (1982) presented the results of a series of 307 microelectrode impalements of shrimp (*Macrobrachium rosenbergii*) intestinal epithelial cells from 82 animals bathed in a saline approximating the haemolymph in ion composition and indicated that the mean transapical electrical potential under these conditions was $74{\cdot}42 \pm 0{\cdot}21$ mV (inside negative). Gerencser (1985) measured intracellular Na^+ and Cl^- activities in enterocytes from the marine mollusc *Aplysia*, using ion-selective microelectrodes, and reported their range to be between 10 and 20 mmol l^{-1}. Seawater and hepatopancreatic luminal concentrations of Na^+ and Cl^- are approximately 450–500 mmol l^{-1}. If transapical potential and intracellular Na^+ and Cl^- activities in hepatopancreatic cells and intestinal cells from crustaceans and molluscs are similar, large electrical and ionic gradients exist in these locations which can power the accumulative transport of organic solutes from lumen to cytosol.

The nature of the specific driving force(s) responsible for accumulation of a given nutrient by hepatopancreatic vesicles, and probably by epithelial cells *in vivo*, depends largely upon the charge that the substrate bears at acidic pH, prior to transfer across the brush-border membrane. Sugars such as glucose remain uncharged at physiological pH and are cotransported with Na^+ from lumen to

cytosol (Ahearn *et al.* 1985). In this instance the combination of membrane potential and transmembrane chemical gradient for Na^+ powers 'uphill' sugar transfer. Alanine, lysine and, as reported in this paper, leucine, at acidic pH, are transported as membrane-potential-sensitive cations, independent of Na^+, using only the transmembrane potential as a driving force (Ahearn *et al.* 1986; Ahearn & Clay, 1987*a*). Lastly, glutamate (Ahearn & Clay, 1987*b*) and a portion of leucine transport occur in an electroneutral fashion, independent of the membrane potential, but using a combination of transmembrane Na^+ and Cl^- chemical gradients to power their accumulation.

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