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# Evidence for a single common $Na^+$-dependent transport system for alanine, glutamine, leucine and phenylalanine in brush-border membrane vesicles from bovine kidney

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**(1) The characteristics of the $Na^+$-dependent transport of alanine, glutamine, leucine and phenylalanine were studied in bovine renal brush-border membrane vesicles. (2) Inhibition of the transport of any one of these amino acids by any other was mutually competitive. (3) the $K_i$ value for the inhibition of alanine transport by leucine was similar to the $K_m$ for leucine transport; similar interrelationships existed for the other amino acids. (4) Each amino acid was shown to exchange with each of the other amino acids across the membrane. (5) From these and other results it is concluded that the $Na^+$-dependent transport of these four amino acids is catalysed by a single common transport system.**

## Introduction

The transport of neutral amino acids has been studied in renal brush-border membrane vesicles derived from various species. The transport of alanine [1], phenylalanine [2], glutamine [3,4], proline [5–8], glycine [9] cystine [10,11] and cysteine [12] has been studied in some detail. In general, the transport of neutral amino acids requires the cotransport of $Na^+$, and the $Na^+$-dependent uptake of neutral amino acids is membrane-potential sensitive.

Relatively little work, however, has been carried out in renal brush-border membrane vesicles in order to elucidate the specificity of the transport systems involved in the transport of neutral amino acids [5,9,13]. Mircheff et al. [13] concluded that five separate systems with overlapping specificity exist for the transport of neutral amino acids in rabbit kidney brush-border membrane vesicles. This conclusion was based on analysis of the patterns of inhibition of transport of substrate amino acids by other amino acids added in very large excess. The inhibition of transport of one amino acid by another does not, however, necessarily imply that these amino acids are transported on a common carrier system, since this inhibition can be either competitive or non-competitive. Thus it was decided to reinvestigate the specificity of renal brush-border neutral amino-acid transport in more detail using a wider variety of techniques than used previously.

For this study, it was convenient to use brush-border membrane vesicles from bovine kidney. These vesicles can readily be prepared in large quantity, and exhibit high rates of amino-acid transport. The vesicles can also be stored frozen for prolonged periods with no loss of transport

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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activity. In this investigation the transport of alanine, glutamine, leucine and phenylalanine has been investigated in detail. Since these amino acids are transported by clearly defined transport systems in hepatocytes and many other cell types, they were chosen as substrates in order to allow a comparison between transport systems for neutral amino acids in these different tissues.

In this investigation, a variety of different approaches have been used to analyse the transport of neutral amino acids in kidney brush-border membranes. Evidence is presented that alanine, glutamine, leucine and phenylalanine are transported on a common $Na^+$-dependent carrier system.

## Methods

*Membrane preparation*

Fresh bovine kidney was obtained from the local abattoir and packed in ice. Slices of cortex were homogenised in a Waring Blendor at full speed for 30 s at 4°C in a medium containing 0.3 M D-mannitol, 5 mM EGTA and 12 mM Tris-HCl (pH 7.4). Renal brush-border membrane vesicles were then isolated essentially by the $MgCl_2$ precipitation method of Booth and Kenny [14] as modified by Biber et al. [15]. The final membrane preparation was suspended at a protein concentration of 10–15 mg/ml in a medium containing 0.25 M sucrose, 10 mM potassium-Hepes, 0.2 mM $CaCl_2$ at pH 7.4, ('sucrose medium'), immediately frozen in small aliquots in liquid nitrogen and stored at −20°C. These vesicles retained transport activity for up to 2 months.

The purity of the membranes was routinely checked by assaying alkaline phosphatase activity [16], a marker enzyme for the brush-border membrane. The activity of the basolateral marker enzyme $(Na^+ + K^+)$-ATPase [17] was also assayed in the preparation. The enrichment of alkaline phosphatase activity was 12-fold with respect to the original kidney cortex homogenate, whereas that of the $(Na^+ + K^+)$-ATPase was enriched by a factor of 1.5. These values are consistent with those reported for renal brush-border membrane vesicle preparations from other species, e.g., see Ref. 2, 11, 13. This indicates that the preparation was predominantly one of luminal brush-border membranes.

*Transport assay*

The technique of rapid filtration through a nitrocellulose membrane (Millipore; 0.45 μm) was used to measure the amount of solute trapped in the membrane vesicles. All experiments were performed at room temperature (20°C). The transport reaction was initiated by the addition of 5–10 μl of vesicle suspension containing 0.05–0.1 mg of protein to an equal volume of medium containing the substrate amino acid labelled with $^3H$ or $^{14}C$ at a specific activity of 200 dpm/pmol for $^3H$ or 50 dpm/pmol for $^{14}C$, together with 0.1 M NaCNS or KCNS and 10 mM $MgCl_2$ in sucrose buffer. The reaction was terminated after the appropriate time by the addition of 1 ml of ice-cold 'stop' solution (sucrose buffer containing 0.2 M NaCl). The suspension was immediately filtered and the filter was washed three times with 1 ml of the stop solution and then removed and placed in 10 ml of scintillation fluid (Unisolve E; Koch-Light Laboratories, U.K.). After the filter had dissolved (approx. 30 min) the solution was assayed for radioactivity by liquid scintillation counting. The presence of the NaCNS created an inwardly directed $Na^+$-gradient which allowed maximum rates of electrogenic $Na^+$–amino-acid cotransport. Measurements of $Na^+$-independent amino-acid uptake were performed in the presence of a gradient of KCNS. Details of individual experiments are stated in the legends to the figures.

Protein was assayed by the method of Bradford [18], using bovine albumin (Fraction V) as a standard.

Radiolabelled alanine, glutamine, leucine and phenylalanine were purchased from Amersham.

## Results

*Characteristics of neutral amino-acid transport in bovine kidney brush-border membrane vesicles*

The time course of the uptake of alanine, glutamine, leucine and phenylalanine (at 0.1 mM concentration) by bovine kidney brush-border membrane vesicles is shown in Fig. 1. The apparent uptake extrapolated to zero time was independent of $Na^+$ and was taken to represent bind-

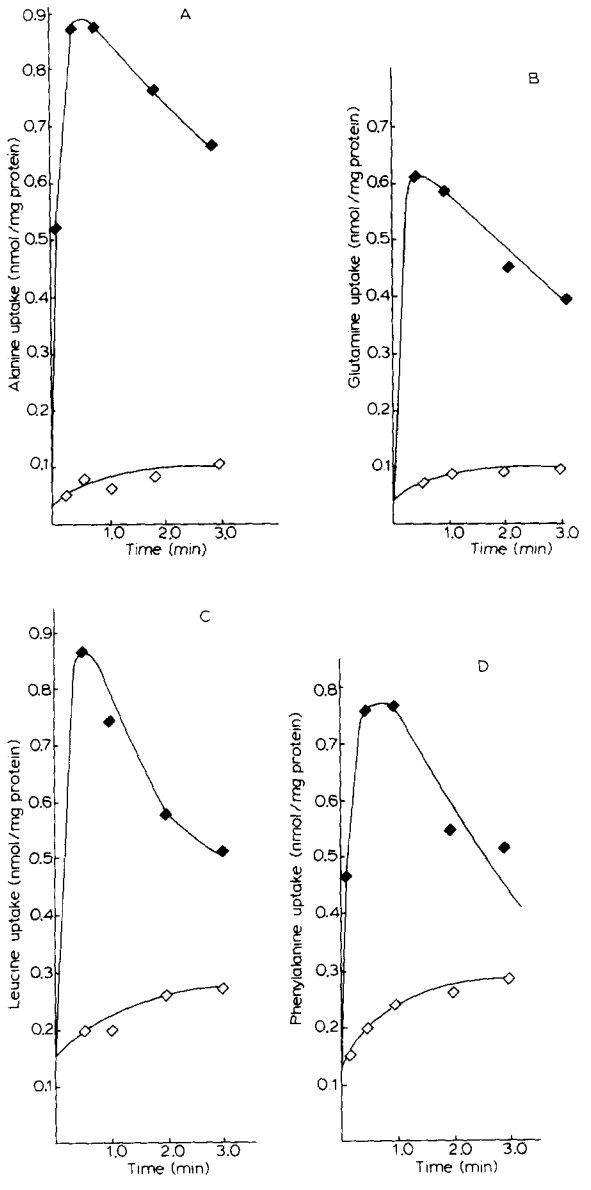


Fig. 1. Time courses of the uptake of (A) alanine, (B) glutamine, (C) leucine and (D) phenylalanine into brush-border membrane vesicles. Membranes were incubated with a final concentration of 0.1 mM of the amino acid shown in sucrose medium containing 5 mM $MgCl_2$ and either 100 mM NaCNS or 100 mM KCNS as shown. Uptake of the amino acid was measured at the times shown. ◆, NaCNS; ◇, KCNS.

ing. As in other such systems, the transport of all these amino acids showed an overshoot which is characteristic of transport driven by an $Na^+$-electrochemical gradient. The uptake in each case was maximum after times between 30 and 60 s. The transport of alanine and glutamine under these conditions was almost entirely $Na^+$-dependent. The transport of both leucine and phenylalanine was largely $Na^+$-dependent, but also exhibited a significant rate in the absence of $Na^+$.

In order to obtain maximum rates of electrogenic $Na^+$–amino-acid cotransport in vesicles it is necessary to perform experiments in the presence of a penetrant anion. Fig. 2 shows that the maximum rate of alanine transport is obtained in the presence of thiocyanate. Chloride, which has been used by others in similar experiments, see, for example Refs. 1, 3, 11, 13, gave lower rates of transport in this system, whereas the less penetrant anion, cyclamate, was not so effective in the charge-compensation of $Na^+$–amino-acid cotransport. It can been seen that the uptake at 10 s

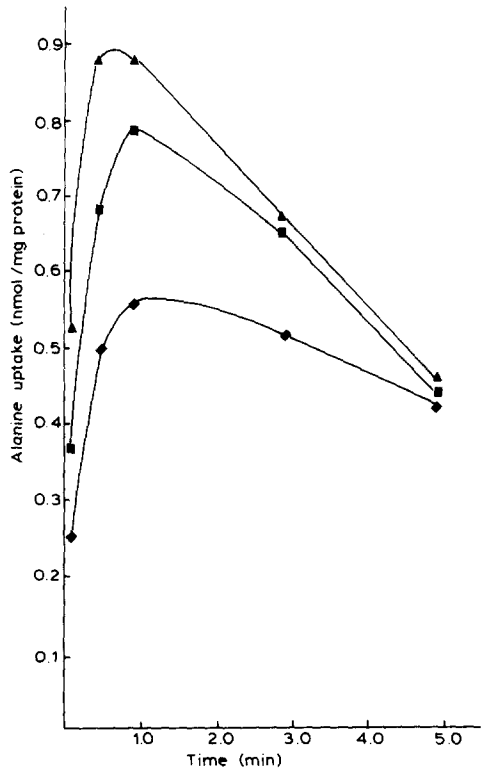


Fig. 2. Anion dependence of $Na^+$-dependent alanine transport. The transport of alanine (0.1 mM) was determined as described in the legend to Fig. 1 in the presence of (▲) 100 mM NaCNS, (■) 100 mM NaCl, or (◆) 100 mM sodium cyclamate.

(which is taken as the initial rate) varied considerably between these three anions; this is in agreement with previous findings for phenylalanine transport [2].

The kinetic parameters of $Na^+$-dependent amino-acid uptake were measured in the presence of thiocyanate after incubation of membranes with different concentrations of amino acids. In a typical experiment the values of $Na^+$-dependent uptake of alanine at 5, 10, 15 and 20 s were 0.34, 0.67, 0.92 and 1.10 nmol/mg protein, respectively. The uptake at 10 s was therefore taken to represent the initial rate. For each amino-acid concentration the rate of transport in the presence of KCNS was also determined; the $Na^+$-dependent rate was calculated as that in the presence of NaCNS minus that in the presence of KCNS. The $Na^+$-independent rate was found to be linear with increasing concentration of each amino acid in the range 0–10 mM. Table I shows the $K_m$ and $V_{max}$ values obtained for the $Na^+$-dependent transport of these amino acids. The $K_m$ value for phenylalanine is similar to that previously determined in rat kidney brush-border membranes [2]. The values for alanine and glutamine are slightly higher than those found for rabbit [1] and rat [3] kidney membranes, respectively. The $V_{max}$ values are in general much higher than those determined in other tissues. This may be due to species differences, and also partly to the use in this investigation of thiocyanate to ensure maximum rates of transport. Both the $K_m$ and the $V_{max}$ values for leucine transport are lower than those of the other amino acids, but the rates of transport at low substrate concentrations are comparable.

*Inhibition of transport by other amino acids*

Table II shows the pattern of inhibition of the initial rate of transport of alanine, leucine, glutamine and phenylalanine (0.1 mM) by a range of other amino acids added at a final concentration of 5 mM. The major feature of these results is the similar degree to which the transport of each of these four amino acids is inhibited by any other amino acid. The stereospecificity of amino-acid transport is clearly shown by the lack of inhibition by the respective D-amino acid. Amino-acid transport is not inhibited by glucose, and methyl 2-aminoisobutyrate, a specific substrate for the A system [19] similarly has little effect.

*Kinetic analysis of inhibition*

Inhibition of the transport of one amino acid by another does not in itself imply that these amino acids are transported on a common carrier system, since the inhibition observed can be either competitive or non-competitive. Therefore, further kinetic studies were undertaken in order to eluci-

TABLE I

KINETIC CONSTANTS OF AMINO-ACID TRANSPORT

Membrane vesicles were incubated with concentrations of amino acids in the range 0.1–10 mM in the presence of NaCNS or KCNS. Initial rates were calculated from the uptake at 10 s. The $Na^+$-dependent rate was taken as the rate in the presence of NaCNS minus that in the presence of KCNS for each concentration of substrate. For each determination the $K_m$ and $V_{max}$ values (±S.E.) were determined from the best fit to a double-reciprocal plot using ten different substrate concentrations.

| Substrate | Membrane preparation | $K_m$ (mM) | $V_{max}$ (nmol·min$^{-1}$·mg$^{-1}$) | Mean $K_m$ (mM) | Mean $V_{max}$ (nmol·min$^{-1}$·mg$^{-1}$) |
|---|---|---|---|---|---|
| Alanine | 1 | 2.55±0.19 | 197 ± 8 | | |
| | 2 | 2.94±0.37 | 119 ± 6 | 2.47 | 136 |
| | 3 | 1.92±0.69 | 93 ± 8 | | |
| Glutamine | 1 | 3.68±1.20 | 195 ±24 | | |
| | 2 | 2.33±0.43 | 190 ±18 | 3.0 | 192 |
| Leucine | 1 | 0.51±0.30 | 35.8± 0.7 | | |
| | 2 | 0.86±0.26 | 28 ± 4 | 0.69 | 32.0 |
| Phenylalanine | 1 | 1.82±0.12 | 108 ± 3 | | |
| | 2 | 2.58±0.10 | 154 ± 6 | 2.2 | 131 |

TABLE II

INHIBITION OF TRANSPORT OF ALANINE, GLUTAMINE, LEUCINE AND PHENYLALANINE BY A RANGE OF AMINO ACIDS

Membranes were incubated in sucrose medium containing (final concentrations) 100 mM NaCNS, 0.1 mM labelled substrate amino acid, and 5 mM inhibitor amino acid. Transport was assayed after 30 s. Controls were performed in media containing either NaCNS or KCNS in the absence of inhibitor. The $Na^+$-dependent rate was taken as the rate in the presence of NaCNS minus that in incubations where NaCNS was replaced by an equal concentration of KCNS. The figures shown are the means of determinations using two membrane preparations; individual measurements agreed to within 10%. n.d., not determined; methyl AIB, methyl 2-aminoisobutyrate.

| Inhibitor (5 mM) | Percentage inhibition of initial rate of $Na^+$-dependent transport | | | |
|---|---|---|---|---|
| Substrate (0.1 mM): | L-alanine | L-glutamine | L-leucine | L-phenylalanine |
| L-Alanine | – | 83 | 75 | 71 |
| L-Glutamine | 82 | – | 72 | 72 |
| L-Leucine | 96 | 96 | – | 98 |
| L-Phenylalanine | 78 | 85 | 77 | – |
| D-Alanine | <10 | n.d. | n.d. | n.d. |
| D-Glutamine | n.d. | <10 | n.d. | n.d. |
| D-Leucine | n.d. | n.d. | 12 | n.d. |
| D-Phenylalanine | n.d. | n.d. | n.d. | <10 |
| L-Lysine | 15 | <10 | <10 | <10 |
| L-Serine | 76 | 75 | 86 | 60 |
| L-Histidine | 35 | 34 | 33 | 45 |
| L-Proline | 47 | 41 | 36 | 38 |
| L-Tryptophan | 72 | 70 | 75 | 74 |
| L-Tyrosine [a] | 21 | 27 | 33 | 32 |
| L-Valine | 90 | 88 | 91 | 88 |
| L-Cysteine | 87 | 92 | 86 | 82 |
| Methyl AIB | <10 | <10 | <10 | <10 |
| D-Glucose | <10 | <10 | <10 | <10 |

[a] 0.5 mM.

date the characteristics of this inhibition. Fig. 3 shows representative Dixon plots of the inhibition of alanine transport by glutamine (Fig. 3A), leucine (Fig. 3B) and phenylalanine (Fig. 3C) and the inhibition of phenylalanine transport by leucine (Fig. 3D). The inhibition of alanine transport by all three of these amino acids was clearly competitive, as was the inhibition of phenylalanine transport by leucine. The $K_i$ values for the inhibition of alanine transport by glutamine (0.9 mM), leucine (0.2 mM), and phenylalanine (1.3 mM) were in reasonable agreement with the $K_m$ values for the transport of these respective amino acids. Similarly, the $K_i$ for inhibition of phenylalanine transport by leucine (0.25 mM) was similar to the $K_m$ for leucine transport. Further experiments (not shown) indicated that glutamine transport was inhibited competitively by alanine ($K_i$ 1.45 mM) and inhibition of glutamine transport by leucine was also competitive ($K_i$ 0.4 mM). Leucine transport was inhibited competitively by phenylalanine ($K_i$ 1.65 mM). In contrast, alanine transport was inhibited non-competitively by glycine ($K_i$ 9.5 mM, results not shown). Glycine has been shown to be transported on a single specific carrier system with a $K_m$ of 1 mM [9].

*Exchange of amino acids across vesicle membranes*

These results are consistent with the postulate that the $Na^+$-dependent transport of these four amino acids is mediated by a single common carrier. If this postulate is correct, it should be possible to demonstrate carrier-mediated exchange of these amino acids across the brush-border membrane. Table III illustrates an experiment designed to investigate this point. Membrane vesicles

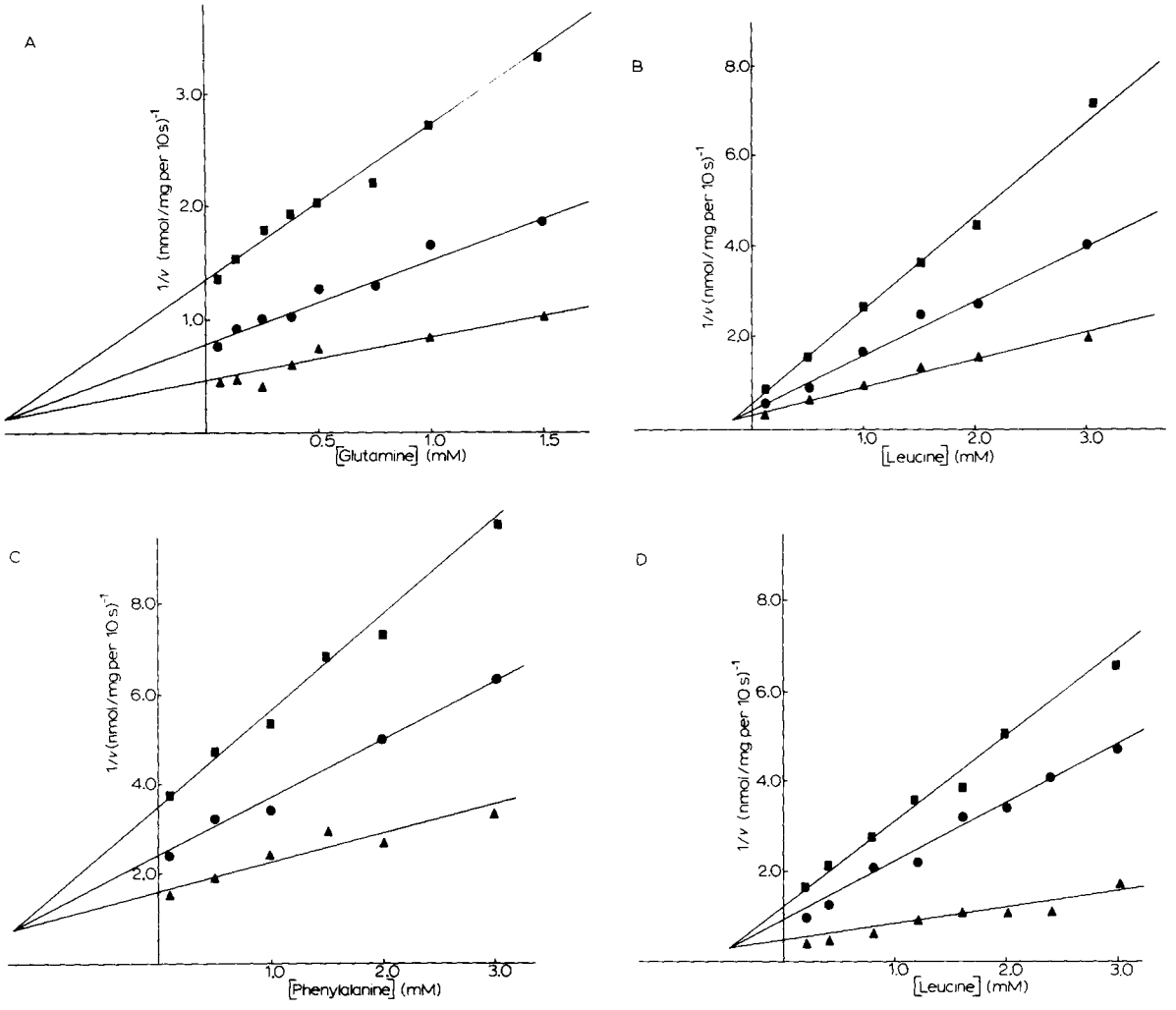


Fig. 3. Inhibition of alanine transport by (A) glutamine; (B) leucine; (C) phenylalanine. Membranes were added to sucrose medium containing 5 mM $MgCl_2$, 100 mM NaCNS, labelled alanine plus unlabelled amino acid as shown. Initial rates were determined at $t = 10$ sec. For each substrate concentration the $Na^+$-independent rate in the absence of inhibitor was subtracted. The lines drawn represent the best fit to the experimental values as determined by the method of least squares. ■, 0.1 mM; ●, 0.25 mM; ▲, 0.5 mM alanine. (D) Inhibition of phenylalanine transport by leucine. Details of the experiment are those shown in (A) with alanine replaced by phenylalanine and glutamine replaced by leucine. ■, 0.1 mM; ●, 0.25 mM; ▲, 0.5 mM phenylalanine.

were preloaded by preincubation with 0.1 mM labelled amino acid for 30 s in the presence of NaCNS before addition of a second unlabelled amino acid at a concentration of 5 mM. The efflux of isotope was assayed after a further 30 s. The Table shows that each amino acid exchanges with each of the other three amino acids. Glucose, which is also transported together with $Na^+$, failed to exchange with any of the amino acids, indicating that the exchange observed was not a non-specific effect due to $Na^+$-cycling.

*Other properties of amino-acid transport*

In various other systems, it is possible to differentiate between different amino-acid transport systems by the criterion of pH dependence. Fig. 4 shows the pH-dependence of the transport of alanine. The transport of the other amino acids

TABLE III

EXCHANGE OF AMINO ACIDS ACROSS THE BRUSH-BORDER MEMBRANE

Brush-border membrane vesicles preloaded by incubation for 30 s with 0.1 mM labelled amino acid in the presence of NaCNS in a total volume of 0.015 ml. Exchange was then initiated by the addition of 5 μl of 20 mM unlabelled amino acid to give a final concentration of 5 mM. Transport was terminated after a further 30 s. In control experiments, 5 μl of water was added instead of the unlabelled amino acid. The results are expressed as the percentage of labelled amino acid retained in the vesicles, taking the value in the control experiment to represent 100%. The values shown are the mean S.E. for the number of observations shown in brackets. Where no S.E. is given, the values are the mean of results obtained in two experiments; these differed by less than 10%. Addition of D-glucose (5 mM final concentration) had no effect on the percentage of any of the amino-acid substrates retained in the vesicles under these conditions.

| Addition | Percentage of amino acid retained in vesicles | | | |
|---|---|---|---|---|
| Substrate: | alanine | glutamine | leucine | phenylalanine |
| None | 100 | 100 | 100 | 100 |
| Alanine | 35.5 ± 3.3 (6) | 36.0 | 43.5 | 49.7 ± 4.7 (3) |
| Glutamine | 36.8 ± 2.7 (5) | 42.0 ± 6.6 (3) | 44.5 | 56.7 ± 6.7 (3) |
| Leucine | 44.5 ± 5.1 (4) | 51.7 ± 4.7 (4) | 47.5 | 67.3 ± 2.7 (3) |
| Phenylalanine | 33.5 ± 5.5 (4) | 49.0 | 48.5 | 48.3 ± 4.1 (3) |

showed a similar pH-dependence. Taking the rate for each amino acid at pH 7.3 to be 100, the rates for alanine, glutamine leucine and phenylalanine transport at pH 6.0 were 62, 64, 72, 59, and at pH 8.0 were 88, 87, 93, 91, respectively. The shape of the pH-dependence curve for glucose transport

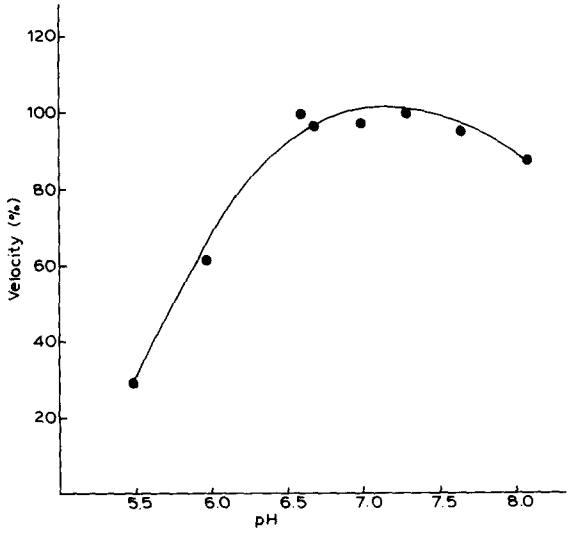


Fig. 4. pH Dependence of alanine transport. Velocity is expressed as a percentage of that at pH 7.3. Vesicles were incubated in sucrose medium containing 10 mM $MgCl_2$, 0.1 mM alanine, 100 mM NaCNS and a further 25 mM potassium-Hepes at the pH values shown. Alanine uptake was measured after 10 s.

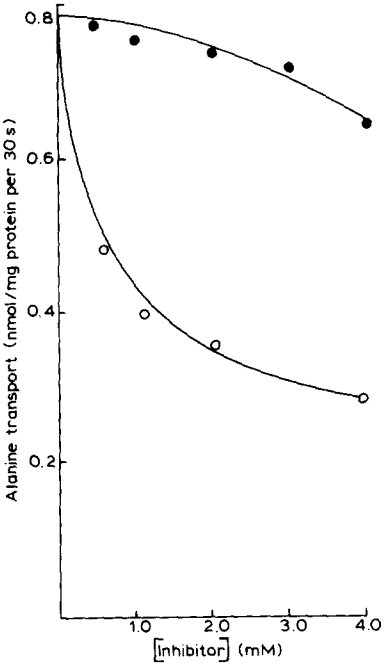


Fig. 5. Inhibition of alanine transport by sulphydryl-blocking reagents. Vesicles were preincubated for 5 min with the concentrations of inhibitors shown on the diagram. Transport was initiated by the addition of an equal volume of sucrose medium containing 0.2 mM labelled alanine, 200 mM NaCNS, and 10 mM $MgCl_2$ and terminated after 30 s. (○, *N*-phenylmaleimide; ●, *N*-ethylmaleimide. The uptake when KCNS was substituted for NaCNS in the absence of inhibitor was 0.1 nmol/mg protein.

was much flatter, with only a 20% increase between pH 5.6 and 8 and no defined pH optimum (results not shown).

Fig. 5 shows that the transport of alanine is strongly inhibited by the sulphydryl-blocking regent *N*-phenylmaleimide, but unusually not by the closely related compound *N*-ethylmaleimide. Similar results were obtained for the inhibition of transport of the other amino acids tested; 1 mM *N*-phenylmaleimide inhibited the $Na^+$-dependent transport of glutamine, leucine and phenylalanine (0.1 mM) by 47%, 45% and 50%, respectively, but no appreciable inhibition was observed with 5 mM *N*-ethylmaleimide. Glucose transport was inhibited by both *N*-phenylmaleimide (half-maximum inhibition at 1.4 mM) and *N*-ethylmaleimide (half-maximum at 3 mM), showing that the inhibition of amino -acid transport is not due to non-specific membrane damage.

## Discussion

The results presented in this paper are consistent with the conclusion that a single transport system mediates the $Na^+$-dependent transport of alanine, glutamine, leucine and phenylalanine in bovine renal brush-border membrane vesicles. This conclusion is supported by the following experimental data which are criteria for the existence of a common carrier for two or more amino acids according to Heinz [20]: (a) the transport of these four amino acids is mutually competitive; (b) the $K_m$ for the $Na^+$-dependent transport of leucine is similar within experimental error to the $K_i$ for the inhibition by leucine of the transport of alanine, glutamine and phenylalanine; – similar interrelationships apply for each amino acid studied; (c) amino acids which inhibit the transport of alanine inhibit the transport of glutamine, leucine and phenylalanine to a similar extent. In addition, carrier-mediated exchange occurs only for those amino acids transported on a common transport system [21–23]. Finally, the pH-dependency of the transport of all four amino acids is identical, as is the sensitivity to inhibition by *N*-phenylmaleimide. This transport system has an unusually broad specificity in that it can catalyse the transport of branched-chain and aromatic amino acids as well as hydrophilic amino acids such as alanine. It presumably does not catalyse the transport of lysine, since lysine did not inhibit the transport of the other amino acids.

In bovine brush-border membrane vesicles, the $Na^+$-dependent pathway accounts for practically all the transport of alanine and glutamine at physiological substrate concentrations. The transport of leucine and phenylalanine exhibited a minor $Na^+$-insensitive component of transport, which was not saturated at substrate concentrations up to 10 mM. This $Na^+$-dependent transport may be attributed either to a transport system with a high $K_m$, or to diffusion of these hydrophobic amino acids across the membrane.

In a previous investigation, Mircheff et al. [13] concluded that a multiplicity of $Na^+$-dependent transport systems with overlapping specificity existed in rabbit renal brush-border membranes. However, these experiments were done by using concentrations of amino acids in the range 0–10 $\mu$M, and measuring inhibition by other amino acids added in the range 25–100 mM. Such a discrepancy in substrate and inhibitor concentrations can lead to non-specific inhibitory effects. Further, no evidence for competitive inhibition of the transport of one amino acid by another was presented. In the present work, titrations were performed using a range of both inhibitor and substrate concentrations, linear Dixon plots were obtained, and it was shown that the transport of the amino acids tested was mutually competitive. Thus, no evidence for multiple pathways for the $Na^+$-dependent transport of neutral amino acids in bovine renal brush-border vesicles was found; if such pathways exist, it is concluded that they contribute to the transport of neutral amino acids to only a minor extent.

Parallels have been drawn between amino-acid transport systems in renal and intestinal brush-border membranes [24]. The so-called neutral brush-border (NBB) transport system which exists in the latter has a wide specificity for neutral amino acids and is not inhibited by methyl aminoisobutyrate. In these respects, it resembles the renal system identified in the present work. It has also been shown that alanine and glutamine are transported on the same carrier system in isolated enterocytes [25].

The present work further differentiates the

amino-acid transport systems in renal brush-border membranes from the well-studied systems in liver, ascites cells and a number of other non-epithelial cell types [26]. For example, in hepatocytes the transport of branched-chain [27] and aromatic amino acids [28] is $Na^+$-independent. Alanine and glutamine are transported on separate carriers in hepatocytes [29], and the inhibition of alanine transport by glutamine is non-competitive [30]. Alanine transport in hepatocytes is partially inhibited by methyl 2-aminoisobutyrate and is sensitive to *N*-ethylmaleimide [31]. In contrast, the transport system identified in renal brush-border membranes catalyses the $Na^+$-linked uptake of leucine and phenylalanine together with that of both alanine and glutamine. The carrier is insensitive to inhibition by methyl aminoisobutyrate and is not inhibited by *N*-ethylmaleimide.

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