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# PHENYLALANINE UPTAKE IN ISOLATED RENAL BRUSH BORDER VESICLES

J. EVERS, H. MURER and R. KINNE

*Max-Planck-Institut für Biophysik, Frankfurt/M. (G.F.R.)*

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## SUMMARY

The uptake of L-phenylalanine into brush border microvilli vesicles and basolateral plasma membrane vesicles isolated from rat kidney cortex by differential centrifugation and free flow electrophoresis was investigated using filtration techniques.

Brush border microvilli but not basolateral plasma membrane vesicles take up L-phenylalanine by an $Na^+$-dependent, saturable transport system. The apparent affinity of the transport system for L-phenylalanine is 6.1 mM at 100 mM $Na^+$ and for $Na^+$ 13 mM at 1 mM L-phenylalanine. Reduction of the $Na^+$ concentration reduces the apparent affinity of the transport system for L-phenylalanine but does not alter the maximum velocity.

In the presence of an electrochemical potential difference for $Na^+$ across the membrane ($\eta_{Na_o} > \eta_{Na_i}$) the brush border microvilli accumulate transiently L-phenylalanine over the concentration in the incubation medium (overshoot phenomenon). This overshoot and the initial rate of uptake are markedly increased when the intravesicular space is rendered electrically more negative by membrane diffusion potentials induced by the use of highly permeant anions, of valinomycin in the presence of an outwardly directed $K^+$ gradient and of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in the presence of an outward-directed proton gradient.

These results indicate that the entry of L-phenylalanine across the brush border membrane into the proximal tubular epithelial cells involves cotransport with $Na^+$ and is dependent on the concentration difference of the amino acid, on the concentration difference of $Na^+$ and on the electrical potential difference. The exit of L-phenylalanine across the basolateral plasma membranes is $Na^+$-independent and probably involves facilitated diffusion.

## INTRODUCTION

$Na^+$-dependent amino acid transport is observed in non-polarized cells such as the pigeon erythrocytes [1–7] and Ehrlich ascites tumor cells [8–14] as well

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; CFCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MES, 2-(*N*-morpholino) ethane sulphonic acid.

as in polarized epithelial cells such as the small intestine [15–20]. In the kidney it has been known for a long time that the accumulation of amino acids in renal cortex slices requires $Na^+$ [21–27]. However, only recently could it be demonstrated that the transepithelial transport of amino acids is also influenced by the ambient $Na^+$ concentration [28].

These observations led to the assumption that the mechanism of active amino acid transport by the tubular epithelium is similar to that in the small intestine. In this epithelium, as originally proposed by Crane [29, 30] for sugars and further substantiated by Schultz and Curran [19], the transfer of amino acids across the brush border membrane is linked to the entry of $Na^+$ into the cell via a cotransport system. The transfer across the basolateral plasma membranes, however, is not influenced by $Na^+$. Thereby the energy for the active transepithelial transport and for the intracellular accumulation is thought to be provided by the electrochemical potental difference for $Na^+$ across the brush border membrane, which is present in vivo, because the intracellular $Na^+$ concentration is lower than the extracellular concentration and because the electrical potential across the brush border membrane is cell inside negative.

In the present study the influence of $Na^+$ on the transport of L-phenylalanine across the two cell borders was investigated by studying the transport properties of isolated plasma membrane vesicles derived from the luminal and contraluminal surface of renal proximal tubular cells. The results obtained indicate that the renal brush border membrane contains an $Na^+$/L-phenylalanine cotransport system, which in the absence of metabolic energy is capable of accumulating L-phenylalanine, provided that an electrochemical potential difference for $Na^+$ is present across the membrane. Thus, as already demonstrated for D-glucose, similar mechanisms for transepithelial transport seem to operate in the renal proximal tubule and in the small intestine.

## MATERIALS AND METHODS

### *Membrane purification*

Partially purified renal membranes were obtained by differential centrifugation from rat kidney following homogenization in isotonic sucrose as previously described [31]. These membranes were then separated into brush border and basolateral membrane fractions using the Desaga FF4 free-flow electrophoresis machine as detailed before [32].

### *Criteria of purity*

The fractions were routinely assayed as previously described for enzymes shown to be characteristic of brush border microvilli, basolateral membranes, mitochondria and endoplasmic reticulum, namely alkaline phosphatase, $(Na^+ + K^+)$-ATPase, succinate dehydrogenase and glucose-6-phosphatase [32]. Protein was determined after precipitation of the membranes with 10 % ice-cold trichloroacetic acid by the Lowry procedure, with bovine serum albumin as a standard [33]. The brush border fraction showed an 11-fold enrichment of alkaline phosphatase and a 2-fold enrichment of $(Na^+ + K^+)$-ATPase compared to the starting material, whereas in the basolateral plasma membrane fraction the specific activity of $(Na^+ + K^+)$-ATPase

was 25 times higher and that of the alkaline phosphatase, 2.5 times higher than in the rat kidney cortex homogenate.

*Transport studies*

The membrane fractions obtained after electrophoretic separation were suspended by homogenization with a teflon glass homogenizer (10 strokes, 1200 rev./min) in 15 ml of a solution containing 100 mM mannitol, 20 mM Tris/HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer (pH 7.4) and centrifuged for 20 min at 30 000 $\times g$ at 4 °C. The pellets were resuspended in 1 ml of the same buffer using a syringe fitted with a fine needle, centrifuged once more, resuspended in the same solution and diluted to a protein concentration of about 10 mg/ml. The incubation medium contained, unless otherwise stated, 100 mM mannitol, 100 mM NaCl, 20 mM Tris/HEPES buffer (pH 7.4) and 1 mM L-[$^3$H]phenylalanine (16.1 Ci/mmol). When the stereospecificity of the transport was studied 1 mM D-[$^{14}$C]phenylalanine (24.6 Ci/mol) was used. Usually each sample contained 5$\mu$Ci/100$\mu$l of the isotope. The stop solution contained 100 mM mannitol, 20 mM Tris/HEPES and 180 mM NaCl. Osmotic pressure was varied by adding sucrose to the medium. In the latter experiments the osmolarity of the stop solution was increased by the addition of NaCl.

Uptake experiments were carried out in 1 ml disposable Eppendorf tubes according to the method of Hopfer et al. [34]. A portion of 20 $\mu$l of membrane suspension was added at zero to 100 $\mu$l of incubation medium at appropriate temperature and pH (usually 25 °C, pH 7.6). Uptake was stopped by withdrawing 20 $\mu$l of the incubation mixture and adding this to 1 ml of ice-cold stopping solution. The resultant suspension was rapidly filtered through a millipore filter (HAWP, 0.45 $\mu$m) and washed once with 3 ml of ice-cold stopping solution, the total time being 20 s. The filter was dried and counted in a Packard liquid scintillation counter in Instagel® (Packard). A medium sample was also counted with each experiment.

In all cases, incubation of membranes denatured by boiling for 1.5 min or filtration of membranes added to the stop solution directly before addition of 20 $\mu$l of radioactive incubation medium reduced the radioactivity retained to less than 10 % of the equilibrium value.

*Analysis of intravesicular content*

Membranes were incubated with an incubation medium as described above for 2 and 20 min and filtered on millipore filters. The filters were extracted with 3 ml distilled water for 24 h at 4 °C, the extract centrifuged and the supernatant concentrated. Separation of potential products of L-phenylalanine was carried out by thin-layer chromatography on Silica gel (Macherey and Nagel, Düren) using butanol/acetic acid/$H_2O$ (78 : 12 : 10, by vol.) as solvent. Controls were run in which the radioactive L-phenylalanine was added subsequent to the incubation. After separation the chromatograms were cut into 1 cm sections and the sections were counted.

*Materials*

All inorganic chemicals were purchased from Merck (Darmstadt, Germany) and were of the highest purity available. HEPES buffer, 2-(*N*-morpholino)ethanesulphonic acid buffer and valinomycin were obtained from Serva International

(Heidelberg, Germany). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CFCCP) was a product of Boehringer (Mannheim, Germany).

*Calculations*

The amount of medium radioactivity bound to the filters in the absence of membranes in the incubation medium was always lower than 10 % of the equilibrium value obtained in the presence of membranes and was found to be constant under all experimental conditions. No correction was made for this blank. The amount of radioactivity found in the membranes was converted into mol of phenylalanine by the use of the specific activity of the incubation medium assuming equal specific activity inside and outside the membrane vesicle.

## RESULTS

*Phenylalanine uptake by renal plasma membrane vesicles*

*General properties.* Fig. 1 shows the typical features of phenylalanine uptake by isolated brush border microvilli vesicles. Firstly, the uptake is stereospecific. If brush border membrane vesicles containing 100 mM mannitol and 20 mM Tris/HEPES (pH 7.4) are incubated in a medium containing in addition 100 mM NaCl,

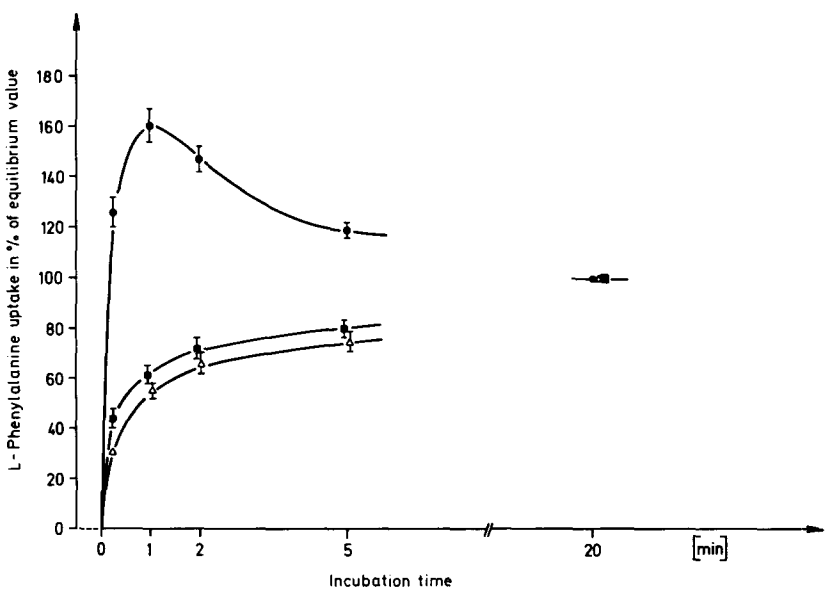



Fig. 1. Uptake of L-phenylalanine (●, ■) and D-phenylalanine (△) by isolated renal brush border microvilli vesicles inthe presence (●, △) and absence (■) of sodium. The vesicles contained 100 mM mannitol and 20 mM Tris/HEPES buffer (pH 7.4) and were incubated in a medium containing in addition 1 mM L-[$^3$H]phenylalanine, 1 mM D-[$^{14}$C]phenylalanine and 100 mM NaCl or KCl, respectively. The values given represent mean values ±S.E.M. derived from five experiments and are expressed as percent of the uptake observed after 20 min. This value was identical under all incubation conditions and represents uptake at equilibrium.

1 mM L-phenylalanine and 1 mM D-phenylalanine; the initial rate* of L-phenylalanine uptake is about 4 times faster than the rate of D-phenylalanine uptake. This difference in the initial uptake is reduced to about 1.5 if $Na^+$ is replaced by $K^+$. Secondly, the uptake of L-phenylalanine is $Na^+$-dependent. In the presence of $Na^+$, compared to the presence of $K^+$ or 200 mM mannitol (not shown), the initial uptake of L-phenylalanine is stimulated three times and an overshoot is observed, i.e. the amount of L-phenylalanine present in the vesicles transiently exceeds the equilibrium value, which is reached after 20 min and is identical for D-phenylalanine and L-phenylalanine under all experimental conditions. D-Phenylalanine uptake is not influenced by the replacement of $Na^+$ by $K^+$. Thus two ways of L-phenylalanine uptake into the microvilli vesicles can be distinguished. One, represented by the D-phenylalanine uptake curve, shows no stereospecificity, no $Na^+$-dependence and no overshoot, and thus might well involve simple diffusion. Another system transports L-phenylalanine but not D-phenylalanine, is influenced by $Na^+$ and gives rise to an overshoot phenomenon. This latter transport system was studied in more detail with regard to its interaction with the amino acid and with cations.

Before proceeding to these questions two additional points should be made. The first concerns the cellular localization of the stereospecific, $Na^+$-dependent L-phenylalanine transport system. As shown in Fig. 2 there is only a slight stimulation of the initial L-phenylalanine uptake into basolateral plasma membrane vesicles by $Na^+$ (about 36 %, compared to 360 % in the brush border membrane vesicles) and no overshoot is observed. Since as judged by the enzymatic analysis the basolateral

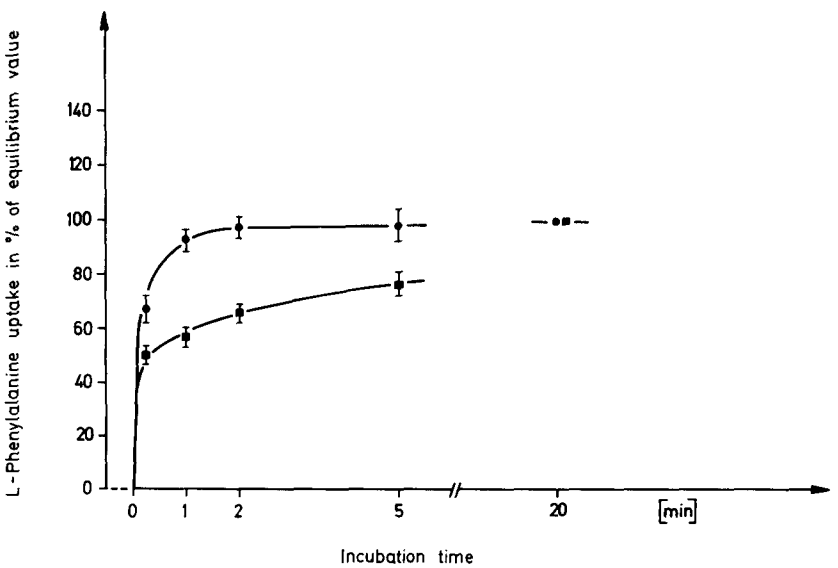



Fig. 2. Uptake of L-phenylalanine by isolated basolateral plasma membrane vesicles. (●) Uptake in the presence of 100 mM NaCl; (■) uptake in the presence of 100 mM KCl in the incubation medium. Other conditions as described in Fig. 1.

* Initial uptake refers to the amount of amino acid taken up by the vesicles during the first 15 s of incubation.

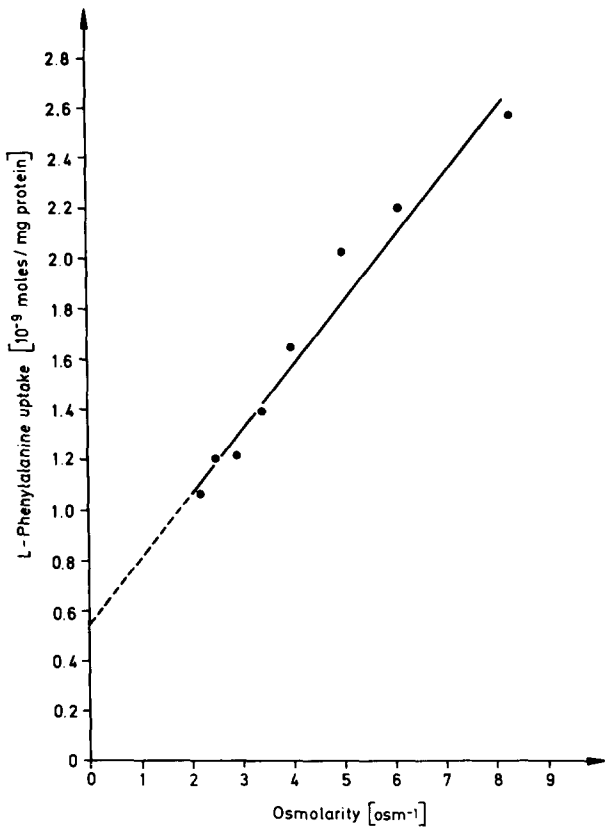
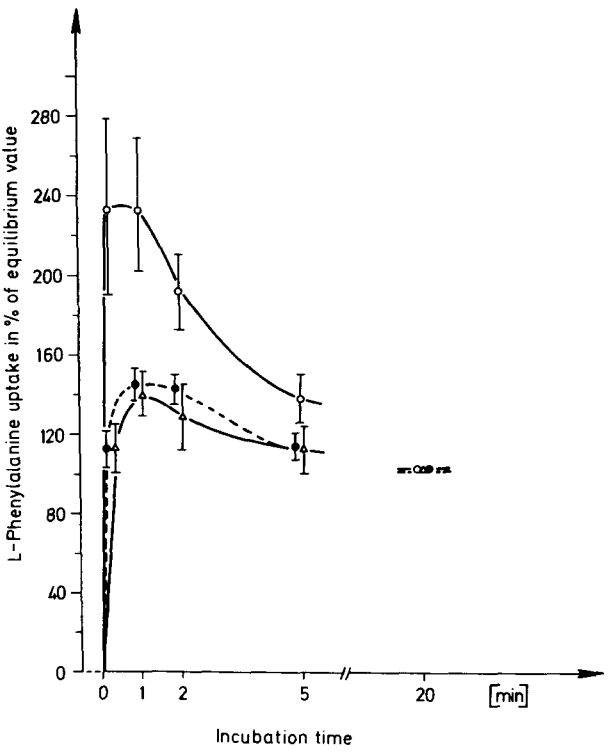



Fig. 3. Influence of increasing osmolarity on the L-phenylalanine uptake by isolated renal brush border microvilli vesicles. The uptake was determined in the presence of 100 mM NaCl and increasing amounts of sucrose in the incubation medium. The values given represent equilibrium values obtained after 20 min of incubation.

plasma membrane fraction still contains some microvilli membranes (see Methods), the $Na^+$ stimulation can most probably be attributed to a small but significant cross-contamination of the basolateral plasma membranes with the brush border microvilli. Thus the $Na^+$-dependent L-phenylalanine transport system seems to be present only in the brush border membranes of the proximal tubule epithelial cell.

Furthermore it had to be clarified whether the uptake of L-phenylalanine by the microvilli membranes represents incorporation into the membranes, adsorption to membrane components or transport into an intravesicular space. The contribution of the former two processes to the results seems to be very small. As shown in Fig. 3, the amount taken up by the vesicles after 20 min of incubation decreases drastically if the osmolarity of the incubation medium is increased by the addition of sucrose. Since, in the range of osmolarity studied, only the intravesicular space should be sensitive to changes in osmolarity, because it is surrounded by a membrane which is almost impermeable to sucrose, the results indicate that mainly transport into an osmotically reactive intravesicular space, rather than binding or incorpora-

A

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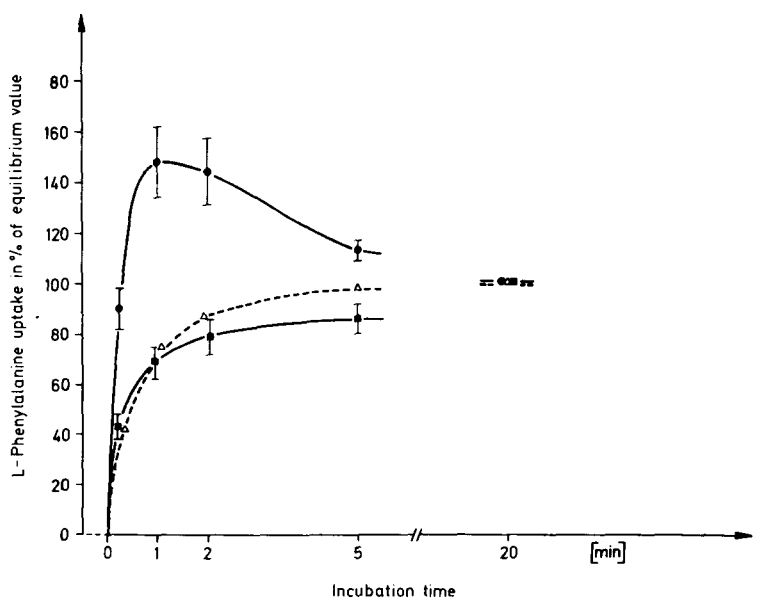


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tion, is measured under the experimental conditions used. It should also be noted that analysis of the intravesicular content after 2 and 20 min of incubation in $Na^+$ and $K^+$ media revealed that more than 90 % of the label found intravesicularly had an $R_F$ value identical to that of L-phenylalanine.

*Interaction of the transport system with* L-*phenylalanine.* The stereospecificity of the phenylalanine uptake demonstrated already in Fig. 1 can also be tested when the potency of the two stereoisomers to elicit trans-stimulation is compared. As shown in Figs. 4A and 4B, preloading of the brush border vesicles with 6 mM L-phenylalanine stimulated the initial uptake of L-phenylalanine both in the presence of

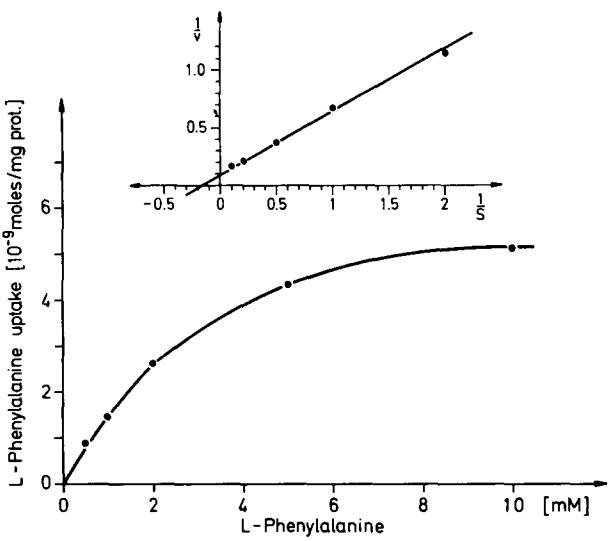


Fig. 5. Influence of L-phenylalanine concentration in the incubation medium on L-phenylalanine uptake by isolated renal brush border microvillus vesicles. The incubation medium contained 100 mM mannitol, 20 mM Tris/HEPES, 100 mM NaCl and the concentration of L-[$^3$H]phenylalanine indicated in the figure. The amount taken up by the vesicles after 15 s is given. The values have been corrected for the $Na^+$-independent uptake by subtraction of the uptake observed with D-phenylalanine.

Fig. 4. (A) Trans-stimulation of L-phenylalanine uptake into isolated renal brush border vesicles in the presence of $Na^+$. The membranes were preincubated for 30 min at 25 °C in a medium containing 100 mM mannitol, 20 mM Tris/HEPES (pH 7.4) (●), 100 mM mannitol, 20 mM Tris/HEPES and 6 mM L-phenylalanine (○) and 100 mM mannitol, 20 mM Tris/HEPES and 6 mM D-phenylalanine (△). Uptake was determined in a medium composed of 100 mM mannitol, 100 mM NaCl (4A), and a final concentration of 1 mM L-[$^3$H]phenylalanine. The values given represent mean values ±S.E.M. derived from four experiments and are expressed as percent of the uptake at equilibrium. (B) Trans-stimulation of L-phenylalanine uptake into isolated renal brush border vesicles in the presence of $K^+$. The membranes were preincubated for 30 min at 25 °C in a medium containing 100 mM mannitol, 20 mM Tris/HEPES (■), 100 mM mannitol, 20 mM Tris/HEPES and 6 mM L-phenylalanine (●) and 100 mM mannitol, 20 mM Tris/HEPES and 6 mM D-phenylalanine (△). Uptake was determined in a medium composed of 100 mM mannitol, 100 mM KCl and a final concentration of 1 mM L-[$^3$H]phenylalanine. The values given represent mean values ±S.E.M. derived from four experiments and are expressed as percent of the uptake at equilibrium.

TABLE I

Effect of $Na^+$ on the kinetic constants of L-phenylalanine uptake by isolated renal brush border microvillus membranes. The results of two experiments are given; they have been calculated from Eadie-Hofstee plots by the least square method. The different $V$ values are due to a different membrane concentration

| $[Na^+]$ in incubation medium | $K_m$ (mM) | $V$ (pmol/min) |
|---|---|---|
| 100 | 2.46 | 2487 |
| 10 | 4.15 | 2616 |
| 100 | 3.66 | 9228 |
| 10 | 5.14 | 10358 |

$Na^+$ and in the presence of $K^+$ by approx. 100 %. This was not observed when the vesicles were preloaded with D-phenylalanine. The finding of trans-stimulation in the absence of $Na^+$ confirms the impression gained in Fig. 1 that also under these conditions part of the L-phenylalanine transport occurs via a stereospecific transport system.

Besides stereospecificity and counterflow, saturability can be demonstrated for L-phenylalanine uptake by brush border microvilli vesicles. Fig. 5 shows an experiment in which the L-phenylalanine concentration of the incubation medium was gradually increased. The values obtained for the uptake during the first 15 s seem to follow simple Michaelis-Menten kinetic and indicate saturability for the $Na^+$-dependent transport system. From the Lineweaver-Burk plot shown in the inset, an apparent Michaelis constant ($K_m$) of 6.1 mM and a maximum velocity ($V$) of 9.1 nmol mg protein per 15 s could be obtained. The influence of $Na^+$ on $K_m$ and $V$ is shown in Table I. If the $Na^+$ concentration in the incubation medium is reduced, the apparent affinity of the L-phenylalanine transport system decreases, whereas the maximum velocity is not altered.

*Interaction of the transport system with cations.* Fig. 6 shows the L-phenylalanine uptake in the presence of 1 mM L-phenylalanine as a function of the $Na^+$ concentration in the incubation medium. Half maximum stimulation of uptake is observed at $13 \pm 0.1$ mequiv. $Na^+$ (mean value $\pm$S.E.M., $n = 7$). The shape of the curve is not simply hyperbolic, but indicates some cooperativity, i.e. that more than one $Na^+$-binding site is present at the transport system. The data can be fitted easily to a line, assuming that two $Na^+$ ions interact with the transport system (see dotted line in Fig. 6). Other monovalent cations such as $K^+$, $Rb^+$, $Cs^+$ and choline do not stimulate L-phenylalanine uptake above the level observed in the presence of isosmolar concentrations of mannitol. Only $Li^+$ seems to be able to mimic partly the action of $Na^+$; the initial L-phenylalanine uptake is stimulated 36 % by $Li^+$, i.e. at identical concentrations $Li^+$ is only about 20 % as potent as $Na^+$ (see Table II). Furthermore, in the presence of $Li^+$ no overshoot is observed. Thus, as in other biological systems, the L-phenylalanine transport system seems not to have an all-or-none preference for a particular cation, but rather possesses some graded specificity for alkali ions.

*Influence of ion gradients and membrane potential on L-phenylalanine uptake by brush border microvilli vesicles.* So far, L-phenylalanine transport across the brush

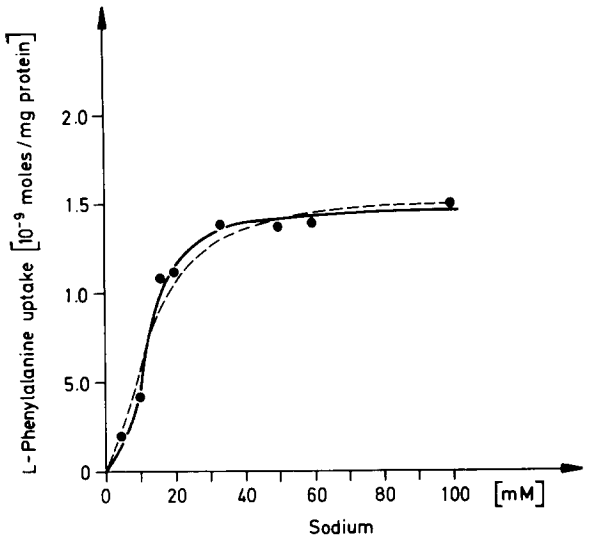


Fig. 6. Influence of $Na^+$ on the L-phenylalanine uptake by isolated renal brush border microvillus vesicles. The incubation medium contained 100 mM mannitol, 20 mM Tris/HEPES (pH 7.4), 1 mM L-[$^3$H]phenylalanine and the $Na^+$ concentration indicated in the figure; $Na^+$ was replaced by choline. The vesicles contained 100 mM mannitol, 20 mM Tris/HEPES and less than 0.1 mM $Na^+$ as determined by flame photometry. Uptake measured after 15 s has been corrected for the uptake observed in $Na^+$-free media. The values represent mean values of eight experiments. The dotted line was calculated assuming that two sodium ions interact with the transport system following the equation.

$$v = \frac{V}{1+\left(\frac{K_{Na^+}}{C_{Na^+}}\right)^2}$$

where $C_{Na^+}$ is the $Na^+$ concentration in the incubation medium and $K_{Na^+}$ is the apparent Michaelis constant for the interaction of $Na^+$ with the transport system.

border membrane has been treated by analysing the initial rates of uptake in analogy to an enzyme reaction which is activated by a positive effector. These considerations indicate that $Na^+$ interacts directly with the L-phenylalanine transport system. However, $Na^+$ does not only increase the initial velocity of L-phenylalanine uptake but also leads to an overshoot phenomenon (see Fig. 1). This overshoot phenomenon most probably represents a transient accumulation of L-phenylalanine inside the brush border membrane vesicles. Osmotic changes of the intravesicular space, which can also produce overshoot-like phenomena, can be excluded because the uptake of D-phenylalanine which enters the same space as L-phenylalanine does not show correspondent changes.

As shown in Table III, the overshoot is not observed if the brush border vesicles have been preincubated in an $Na^+$-containing solution, i.e. if there is no concentration difference for $Na^+$ across the brush border membrane. These data suggest that the electrochemical potential difference of $Na^+$ across the brush border membrane $\Delta\eta_{Na^+}$ provides the driving force for the L-phenylalanine accumulation and that $Na^+$ and phenylalanine fluxes are coupled via an $Na^+$-phenylalanine cotransport system.

TABLE II

Effect of different cation gradients on L-phenylalanine uptake by isolated renal brush border microvillus vesicles. The values are given as percent of the uptake observed in the presence of NaCl and represent mean values from three experiments.

| Salt in the incubation medium | Uptake | |
|---|---|---|
| | After 15 s | After 1 min |
| LiCl | 48.5 | 68.0 |
| NaCl | 100.0 | 100.0 |
| KCl | 38.2 | 42.5 |
| RbCl | 36.1 | 43.8 |
| CsCl | 35.5 | 48.3 |
| Choline chloride | 34.6 | 41.8 |
| Mannitol (200 mM) | 36.0 | 47.5 |

This is especially evident from the experiments shown in Fig. 7. In these experiments, in contrast to the results shown above, no concentration difference for L-phenylalanine existed at the beginning of the experiment, because the membrane vesicles had been preincubated in an L-phenylalanine-containing solution. If then an $Na^+$ gradient was imposed on the membrane by addition of a concentrated salt solution, a transient accumulation of L-phenylalanine in the vesicles was observed. Addition of $K^+$ was without effect.

The electrochemical potential difference $\Delta\eta_{Na^+}$ is composed of the transmembranal concentration difference $\Delta c_{Na^+}$ and the electrical potential difference $\Delta\varphi$ multiplied by the mean $\bar{c}$ transmembranal concentration of $Na^+$, and $zF/RT$. This

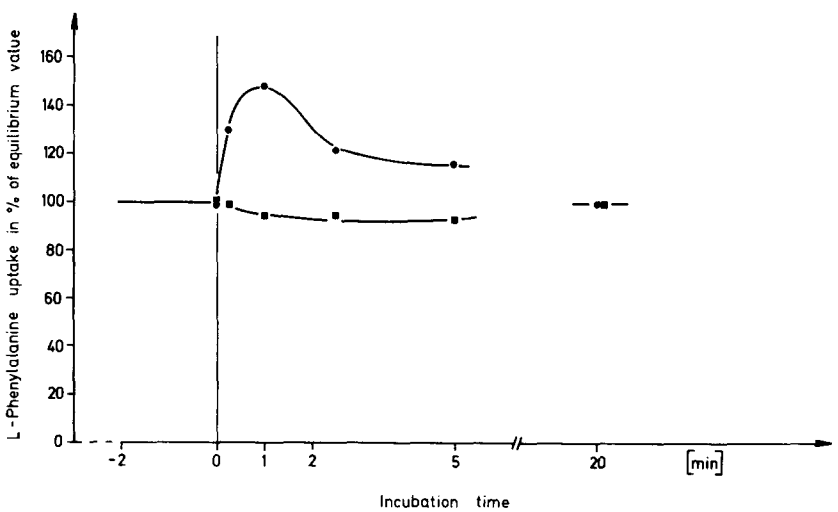


Fig. 7. $Na^+$-induced accumulation of L-phenylalanine in isolated renal brush border microvillus membranes. The membranes were incubated for 30 min at 25 °C in a medium containing 100 mM mannitol, 20 mM Tris/HEPES (pH 7.4) and 1 mM L-[$^3$H]phenylalanine. At the time point indicated, 10 $\mu$l of a 1.1 M solution of NaCl (●) and KCl (■) was added to 100 $\mu$l of the membrane suspension.

TABLE III

Influence of $Na^+$ and $K^+$ on the L-phenylalanine uptake by isolated renal brush border microvillus vesicles under gradient and non-gradient conditions. Under gradient conditions the membrane vesicles had been preincubated in 100 mM mannitol, 20 mM Tris/HEPES for 20 min at 25 °C and the uptake was determined in a medium which contained in addition 1 mM L-[$^3$H]phenylalanine and 100 mM NaCl or KCl, respectively. Under non-gradient conditions the membranes had been preincubated in 100 mM mannitol, 20 mM Tris/HEPES and 100 mM NaCl or KCl, respectively. Mean values ±S.E.M. of at least four experiments are given and are expressed as percent of the equilibrium value.

| | Uptake | | | |
|---|---|---|---|---|
| | Gradient conditions | | Non-gradient conditions | |
| | After 15 s | After 1 min | After 15 s | After 1 min |
| NaCl | 119±15 | 154±19 | 66±14 | 78±12 |
| KCl | 44±13 | 70±18 | 40± 9 | 68±13 |

relation would imply that the L-phenylalanine uptake should depend not only on the concentration difference for $Na^+$ but also on the electrical potential difference. As shown recently [35], the membrane potential of the brush border microvillus vesicles can be manipulated by imposing diffusion potentials on the membrane. Since diffusion potentials depend on the relative mobility of cations and anions, the influence of anion replacement on L-phenylalanine uptake was studied. As shown in Table IV, replacement of $Cl^-$ by a more permeant anion such as $SCN^-$ stimulates the initial uptake and increases the overshoot of L-phenylalanine. In another experimental approach ionophores were used to increase the cation permeability of the membranes specifically in the presence of impermeable anions. Membranes were preloaded with $K_2SO_4$ and the L-phenylalanine uptake in the absence and in the presence of the potassium ionophore valinomycin was examined. As shown in Fig. 8, L-phenylalanine uptake is markedly stimulated by the presence of valinomycin. Similar results are obtained if the uncoupler CFCCP is used under conditions where the pH of the intravesicular space is lower than the pH of the incubation medium (Fig. 9). Under all these experimental conditions it is necessary to assume a hyperpolarization of the

TABLE IV

Effect of anion replacement on the L-phenylalanine uptake by isolated renal brush border microvilli. The incubation medium contained 100 mM mannitol, 20 mM Tris/HEPES, 1 mM L-[$^3$H]phenylalanine, 100 mM $Na^+$ and the anion indicated in the table. The values are expressed as percent of the uptake observed in the presence of $Cl^-$ and represent mean values derived from at least three experiments.

| Anion | Uptake | |
|---|---|---|
| | After 15 s | After 1 min |
| 100 mM $Cl^-$ | 100 | 100 |
| 100 mM $SCN^-$ | 181 | 115 |
| 50 mM $SO_4^{2-}$ | 60 | 68 |

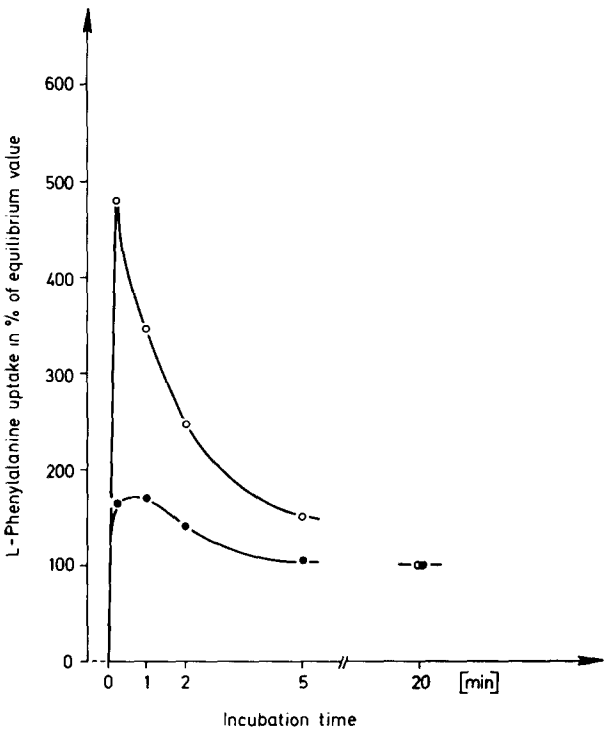


Fig. 8. Influence of $K^+$ diffusion potential on the uptake of L-phenylalanine by isolated renal brush border microvillus vesicles. The membrane vesicles were preloaded with 50 mM $K_2SO_4$, 100 mM mannitol and 20 mM Tris/HEPES. The uptake was measured in a medium containing 50 mM $Na_2SO_4$, 100 mM mannitol, 5 mM $K_2SO_4$, 1 mM L-[$^3$H]phenylalanine and 20 mM Tris/HEPES in the presence (○) and absence (●) of valinomycin.

intravesicular space, which acts as an additional driving force for the accumulation of L-phenylalanine via the $Na^+$-amino acid cotransport system.

If the membrane potential is reversed by the use of $SO_4^{2-}$ instead of $Cl^-$ in the presence of $Na^+$ (Table IV), the rate of uptake is markedly reduced and an uptake curve without overshoot, similar to the uptake after preloading the vesicles with sodium (see Table III) is observed. In this case the electrical term of $\Delta\eta_{Na^+}$ probably compensates the chemical term involving the concentration difference.

It is also possible to design experiments in which no concentration gradient of $Na^+$ is present, and where it can be tested whether the electrical potential difference alone is able to provoke an intravesicular accumulation of L-phenylalanine. For this purpose brush border microvilli vesicles were preincubated in a solution which contained L-phenylalanine and $Na_2SO_4$.

Then a small volume of a concentrated solution of KSCN (permeant anion) or $K_2SO_4$ (impermeant anion) was added to the membrane suspension. As shown in Fig. 10, the permeant anion $SCN^-$ causes a transient stimulation of L-phenylalanine uptake into the membrane vesicles, whereas the impermeant anion is without effect. These results can be explained by the assumption that KSCN gives rise to a diffusion potential that renders the inside of the vesicles electrically more negative. Thereby

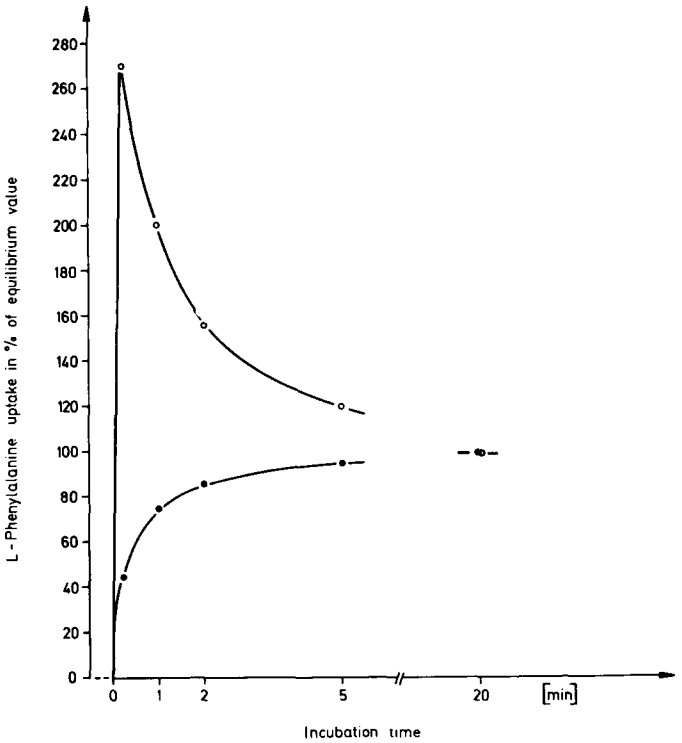


Fig. 9. Influence of a proton diffusion potential on the uptake of L-phenylalanine by isolated renal brush border microvillus vesicles. The membrane vesicles were preloaded with 20 mM MES buffer (pH 5.9) and 100 mM mannitol, the uptake was measured in a medium containing 50 mM $Na_2SO_4$, 100 mM mannitol, 1 mM L-[$^3$H]phenylalanine and 20 mM Tris/HEPES buffer (pH 7.4) in the presence (○) and absence (●) of the uncoupler CFCCP.

an electrical potential difference $\Delta\varphi$ is created which, via an increased $\Delta\eta_{Na^+}$, drives L-phenylalanine into the vesicles.

*Effect of* L-*phenylalanine on* $Na^+$ *fluxes across the brush border membranes*

In a postulated $Na^+$-amino acid cotransport system not only should a stimulation of the amino acid flux by $Na^+$ be demonstrable, but also a stimulation of $Na^+$ flux by the amino acid should be observed. An experiment to test this criterion is shown in Fig. 11. Brush border microvilli were preincubated for 1 h in a solution containing 100 mM mannitol, 5 mM Tris/HEPES, 95 mM KSCN and 5 mM $^{22}$NaSCN. Then a concentrated solution of L-phenylalanine or mannitol was added to the membrane suspension and the changes in the intravesicular $Na^+$ content were monitored. With mannitol, the new equilibrium value, determined by the dilution of the incubation medium after addition of the $Na^+$-free mannitol solution, is reached almost immediately and is maintained (with small variations) during the whole incubation period. The addition of L-phenylalanine elicits a transient accumulation of $^{22}Na^+$ in the brush border membrane vesicles. This result indicates that $Na^+$ entry via the L-phenylalanine-$Na^+$ cotransport system can be driven by a gradient of L-phenylalanine across the brush border membranes.

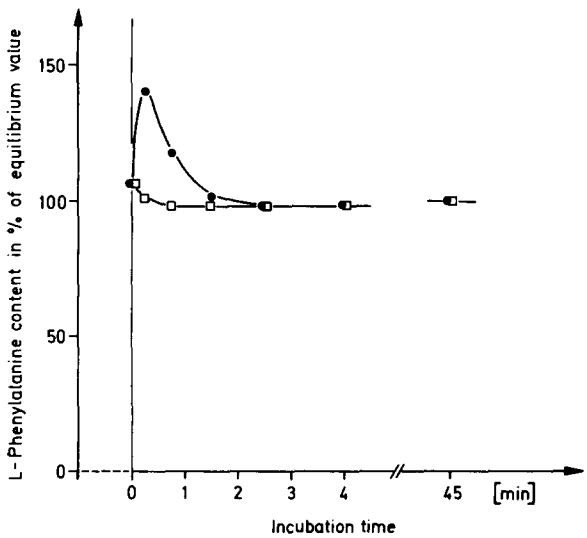


Fig. 10. Accumulation of L-phenylalanine in isolated renal brush border microvillus membranes induced by changes of the membrane potential. Isolated brush border microvilli were incubated for 1 h at 25 °C in a solution containing 100 mM mannitol, 50 mM $Na_2SO_4$, 5 mM Tris/HEPES (pH 7.4) and 1 mM L-[$^3$H]phenylalanine. At zero time 10 $\mu$l of 1.6 M KSCN (●) or 0.8 M $K_2SO_4$ (□) were added to 150 $\mu$l of the membrane suspension. The values are expressed as percent of the new equilibrium reached after 45 min of incubation. They represent mean values derived from four experiments.

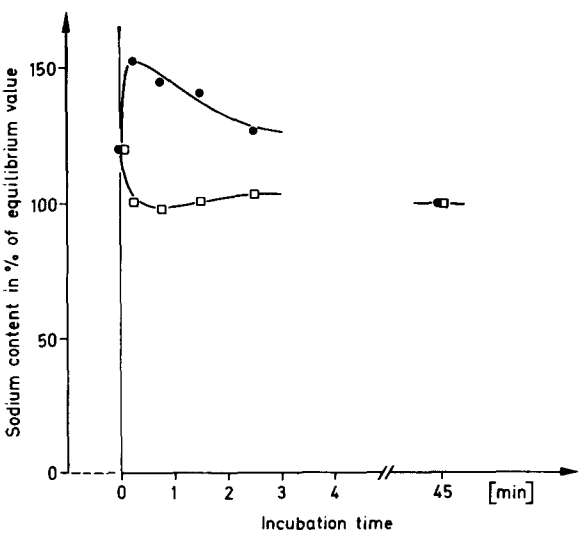


Fig. 11. L-phenylalanine-induced $Na^+$ accumulation in isolated renal brush border microvillus membranes. Isolated brush border microvilli were incubated for 1 h at 25 °C in a solution containing 100 mM mannitol, 5 mM Tris/HEPES (pH 7.4), 95 mM KSCN, 5 mM $^{22}$NaSCN. At zero time, 30 $\mu$l containing 5 mM Tris/HEPES, 75 mM KSCN and 150 mM L-phenylalanine (●) or 150 mM mannitol (□) were added to 150 $\mu$l of the membrane suspension. The values are expressed as percent of the new equilibrium reached after 45 min of incubation. They represent mean values derived from four experiments.

It should be noted in this context that experiments to demonstrate flux coupling (analogous to the influx studies demonstrated for L-phenylalanine above), i.e. in the presence of both an electrochemical potential difference of $Na^+$ and a concentration difference of L-phenylalanine, failed because the entry of $Na^+$ into the brush border microvilli vesicles occurs mainly (more than 90 %) via other systems than the amino acid-$Na^+$ cotransport system.

## DISCUSSION

Our studies on the L-phenylalanine transport by isolated renal brush border membranes indicate that the transfer of the amino acid across the membrane occurs via an $Na^+$-dependent transport system. This transport system most probably catalyses a cotransport of $Na^+$ and amino acids, as indicated by the stimulation of phenylalanine uptake by $Na^+$ and vice versa. Similar systems have been demonstrated in isolated membranes for D-glucose [34–36] and L-alanine [37] in the intestine and for sugars in the kidney [38].

One advantage of studies on isolated membrane vesicles is that the driving forces of the transmembranal transport can be investigated independently from cellular metabolism, because preparations can be obtained which do not contain any glycolytic enzymes or ATP. This, however, seems to hold only for intestinal and renal plasma membrane vesicles isolated according to Hopfer et al. [34] or Kinne et al. [38], respectively. Other renal membrane preparations, for example that of Hillman et al. [39] and that of Johnstone et al. [40] isolated from Ehrlich ascites tumour cells, contain either glycolytic enzymes or ATP and therefore studies on the driving forces are still ambiguous.

As demonstrated above, in the presence of an electrochemical potential difference of sodium ($\Delta\eta_{Na_o} > \Delta\eta_{Na_i}$) the $Na^+$-amino acid cotransport system is able to accumulate phenylalanine inside the vesicles, thereby performing secondary active transport.

Phenomenologically, the coupling between phenylalanine and $Na^+$ flux can be described by the equation [49]

$$J_i = L_{ii}\Delta\eta_i + L_{iNa^+}\Delta\eta_{Na^+}$$

which relates the net flux of L-phenylalanine ($J_i$) to the two driving forces derived from the concentration difference of the substrate ($\Delta\eta_i$) and that derived from the electrochemical potential difference of sodium ($\Delta\eta_{Na^+}$). $L_{ii}$ is a straight coefficient and $L_{iNa^+}$ is the cross-coefficient which expresses the coupling of phenylalanine to $Na^+$. The occurrence of an overshoot of phenylalanine inside the vesicles under conditions where initially an $Na^+$ gradient is present can be explained as follows. At the beginning of the incubation $\Delta\eta_i$ and $\Delta\eta_{Na^+}$ are positive and cause the influx of L-phenylalanine; as soon as the intravesicular L-phenylalanine concentration is equal to the concentration in the medium, $\Delta\eta_i$ becomes zero, but there is still the driving force of $\Delta\eta_{Na^+}$. During the accumulation, $\Delta\eta_i$ becomes inverse, until $\Delta\eta_i$ and $\Delta\eta_{Na^+}$ cancel each other, then an efflux following $\Delta\eta_i$ occurs until finally $\Delta\eta_i$, $\Delta\eta_{Na^+}$ and $J_i$ become zero and the equilibrium is reached. Under the conditions of $Na^+$ preloading, $\Delta\eta_{Na^+}$ is zero; therefore no overshoot is observed.

As already pointed out above, $\Delta\eta_{Na^+}$ is a function of the concentration dif-

ference and the membrane potential. In the vesicular system the membrane potential can easily be modified, and thus its influence on the transport can be studied. The results reported above clearly indicate that the electrical potential across the membrane is one of the determinants of the L-phenylalanine transport. This finding is of general importance because it demonstrates that considerations about the adequacy of ion gradients to support the intracellular accumulation of amino acids should include the membrane potential as one of the driving forces, not only for charged amino acids but also for electrically neutral ones. The involvement of the membrane potential can also explain the observation that in the proximal tubule at an intratubular $Na^+$ concentration of 5 mM still about 50% of the maximum active transport of L-phenylalanine is observed, although under these conditions the $Na^+$ concentration inside the cell is probably higher than in the tubule. For a similar situation, the accumulation of α-aminoisobutyric acid in the presence of inverted $Na^+$ and $K^+$ gradients by Ehrlich ascites tumour cells, it was recently demonstrated by Heinz et al. that the electrical potential difference across the cell membrane was sufficient to account for the accumulation observed [41, 42].

At last some implications of our findings for the cellular basis of transepithelial transport in the kidney should be considered. The comparison of L-phenylalanine uptake by isolated brush border microvilli vesicles and basolateral plasma membrane vesicles provided evidence that the $Na^+$-dependent L-phenylalanine transport system is present predominantly in the brush border membrane. However, no unequivocal hint at the presence of an L-phenylalanine transport system in the basolateral plasma membranes could be obtained, although it has been demonstrated in micropuncture and in microinjection studies that the basal pole of the proximal tubular cell contains specific transport systems for amino acids [43–48]. The reason for this failure is on the one hand that renal basolateral plasma membrane fractions are still contaminated with some brush border microvilli and on the other hand that in contrast to the sugar transport system specific inhibitors for the luminal amino acid transport system are lacking. Moreover, the L-phenylalanine transport system of the brush border membrane is not as strictly $Na^+$-dependent as the glucose transport system. Therefore, even if, for example, trans-stimulation of L-phenylalanine uptake under $Na^+$-free conditions is observed with basolateral plasma membranes this cannot be considered as conclusive evidence for the presence of a specific transport system in these membranes, because brush border membranes show a similar behaviour.

Irrespective of these technical difficulties, the uneven distribution of the L-phenylalanine transport systems in the proximal tubular cell is evident from our studies. The luminal localization of the $Na^+$-L-phenylalanine cotransport system in conjunction with the electrochemical potential difference of $Na^+$ across the brush border membrane enables the renal epithelial cell to perform active transepithelial transport of L-phenylalanine. The same mechanism might underlie the reabsorption of other neutral amino acids.

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