Amino Acids (1995) 8: 247–264

Amino
Acids

Comparative study of the uptake of L-cysteine and L-cystine in the renal proximal tubule

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Accepted December 12, 1994

Summary. 1. The transport mechanisms of L-cysteine and L-cystine by luminal membrane vesicles isolated from either the proximal convoluted part (pars convoluta) or the proximal straight part (pars recta) of rabbit proximal tubuli were examined.

2. The uptake of L-cysteine in pars convoluta is characterized by a single sodium-dependent transport system (Km = 0.58 mM), whereas the sodium-dependent influx of L-cysteine in pars recta proceeds via transport systems with different affinities (Km_1 = 0.03 mM, Km_2 = 5.84 mM). An H^+-gradient enhanced the uptake of L-cysteine in pars recta and only in the presence of a Na^+-gradient.

3. The presence of a Na^+ gradient stimulated the influx of L-cystine in both parts of the nephron, but to a lesser extent than for L-cysteine. In addition a considerable amount of binding of L-cystine to membrane vesicles was observed in both pars convoluta and pars recta.

4. Stoichiometric studies indicated a coupling ratio of 1 Na^+:1 amino acid for the transport components involved in the uptake of L-cysteine and L-cystine along the proximal tubule.

5. Competition experiments demonstrated that neutral α-amino acids inhibited the influx of L-cysteine in the proximal tubule. Basic and acidic amino acids had no effect on uptake of L-cysteine in pars convoluta, whereas a slight inhibitory effect of L-lysine and L-arginine was noted in pars recta.

6. Both basic and neutral amino acids, but not L-glutamate inhibited the uptake of L-cystine in pars convoluta. This was in general also the case in pars recta. However, addition of L-proline did not influenced the uptake of L-cystine, and L-phenylalanine, L-asparagine, L-glutamine, L-leucine and L-methionine only inhibited at a high concentration (5 mM).

Keywords: Amino acids – L-Cysteine – L-Cystine – Na^+-dependent – H^+-dependent – Stoichiometry – Pars convoluta – Pars recta – Rabbit kidney

Introduction

The presence of inherited cystinuria has resulted in numerous studies to delineate the underlying mechanisms of renal tubular reabsorption of L-cystine and L-cysteine. Earlier electrophysiological studies by Samarzija and Frömter (1982a) showed that L-cysteine transport across the rat renal proximal tubular luminal membrane is dependent on sodium, mediated by a single transport process with a net positive charge. Transport studies of L-cysteine in rat renal brush border membrane vesicles (Stieger et al., 1983) confirmed that L-cysteine uptake is strongly stimulated by an inwardly directed sodium gradient and an overshoot phenomenon was observed. However, it was reported that the uptake of L-cysteine is characterized by two transport systems, and that influx of the amino acid is inhibited by L-alanine and L-phenylalanine, but not by L-acidic and L-basic amino acids. By electrophysiological analysis of amino acid transport in rat kidney proximal tubule in vivo Samarzija and Frömter (1982b) showed that the transport system used by basic amino acids does not accept acidic or neutral amino acids with the possible exception of cystine. Experiments, using microperfusion (Völkl and Silbernagl, 1982b) and filtration techniques (McNamara et al., 1981), have demonstrated the existence of two different transport systems for L-cystine. A high affinity system which is shared with basic amino acids, and a low affinity system. Studies using brush border membrane vesicles (McNamara et al., 1981; Biber et al., 1983) have shown that the transport of L-cystine is sodium dependent. The uptake of L-cystine, at low substrate and inhibitor concentration, was found to be inhibited by the basic amino acids, whereas the influx of L-cystine only was reduced by the neutral amino acids L-phenylalanine and L-leucine at higher inhibitor concentrations (Biber et al., 1983). Others have postulated that the transport of L-cystine is inhibited by the neutral amino acids L-alanine and L-phenylalanine (Völkl and Silbernagl, 1982a). McNamara et al. (1981a) have demonstrated that in addition to the dibasic L-amino acids arginine, lysine, and ornithine, the transport of L-cystine is inhibited by the neutral amino acid L-glutamine, whereas phenylalanine, valine, proline and α-aminoisobutyric acid had no effect. However, none of the previous studies were able to inform us, whether there are different luminal transport mechanisms for L-cysteine or L-cystine in the pars recta and pars convoluta of the nephron, even though a multiplicity of transport mechanisms for L-cystine have been proposed in order to explain the different forms of cystinuria (Biber et al., 1983; Cho and Bannai, 1990). Nor has the effect of an inwardly directed pH gradient on the transport of L-cysteine been examined in contrast to previous reports concerning L-cystine (Reynolds et al., 1991). To resolve the complexities resulting from the presence of different transport systems in specialized regions of the proximal tubule, we isolated separately luminal membrane vesicles from pars convoluta and pars recta of rabbit kidney. These studies provide an excellent model for an analysis of the co-transport mechanisms due to the facts that the experimental conditions can be controlled very strictly, and metabolic processes are excluded. This is of great importance, especially in this study,

since the reabsorbed L-cystine is rapidly converted to L-cysteine and vice versa (a conversion that may take place even before or during the uptake process) (Schafer and Watkins, 1984). The results of the present study demonstrate the existence of different transport mechanisms, the effect of Na^+ and changes in pH on the luminal uptake of L-cysteine and L-cystine, the effect of different amino acids on the luminal uptake of L-cysteine and L-cystine, and the stoichiometry in pars convoluta and pars recta.

Materials and methods

Chemicals

All non-radioactive amino acids, Trizma base, Hepes and Mes were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Radiochemicals, L-[^{35}S]-cysteine and L-[^{35}S]-cystine, were purchased from New England Nuclear Corp., Boston, MA, U.S.A. 3.3′-Diethyloxadicarbocyanine iodide was supplied by Eastman Kodak Co., Rochester, NY, U.S.A. All reagents used in this study were of A.R. grade.

Isolation of luminal-membrane vesicles

Male and female rabbits weighing approximately 3–4 kg were killed by a blow to the neck, exanguinated and the kidneys excised. Luminal membrane vesicles were isolated from either pars convoluta ("outer cortex") or pars recta ("outer medulla") of the proximal tubules of rabbit kidney according to the method previously described (Sheikh and Møller, 1987). Briefly, outer cortical tissue was obtained by cutting thin slices, <0.3 mm thick, from the surface of the kidney. Strips of outer medulla approx. 1 mm thick (representing predominantly pars recta) were dissected from outer stripe of the outer medulla. Luminal membrane vesicles from outer cortical and outer medullary tissue were prepared in parallel from the same kidneys, by use of the Ca^{2+}-precipitation procedure (Sheikh and Møller, 1987). Unless otherwise stated, the vesicles were suspended in a solution containing 310 mM mannitol and 15 mM Hepes/Tris buffer, pH 7.5. The protein concentration in the membrane fractions was determined according to Lowry et al. (1951) as modified by Peterson (1977).

Uptake of amino acid by membrane vesicles

The uptake of amino acid by luminal membrane vesicle was studied by the Millipore filtration method as follows: At time zero 20 μl of luminal membrane vesicle suspension was added to 100 μl of incubation medium containing radioactive amino acid. The exact composition of the various incubation media are given in the legends to figures. Transport of amino acid into vesicles was stopped at timed intervals by adding 1 ml ice-cold stop solution, consisting of either 155 mM NaCl or 155 mM KCl dissolved in 15 mM Hepes/Tris buffer (pH 7.5) or in 15 mM Mes/Tris buffer (pH 5.5), in the low pH experiments. The resulting suspension was rapidly filtered through a wet Millipore filter (0.45 μm, HAWP) and washed twice with 2.5 ml of the ice-cold buffer. The filter was dried and radioactivity was counted in a liquid scintillation counter (LKB-Wallac 1218 RackBeta) after addition of 5 ml Filter Count™ (Packard Instrument International SA. Zürich, Switzerland). All experiments were performed at 22 ± 2°C. Correction for nonspecific binding to the filter and membrane vesicles was made by subtracting from all uptake data the value of a blank obtained by addition of ice-cold stop solution to incubation medium and vesicles before they were mixed and filtered. All experiments were repeated at least

three times. Within the same experiment all uptake measurements were performed in triplicate.

Uptake of L-cysteine and L-cystine by vesicle preparations was also examined by a spectrophotometric method with potential-sensitive carbocyanine dye as previously described (Kragh-Hansen, Jørgensen and Sheikh, 1982). For this, 60 μl membrane vesicle suspension was added to 2.4 ml 3.3′-Diethyloxadicarbocyanine iodide, 15 mM Hepes/Tris (pH 7.5), and 155 mM NaCl or 155 mM KCl in an Aminco DW-2a, thermostated at 20°C. The initial intravesicular medium was 310 mM mannitol and 15 mM Hepes/Tris (pH 7.5). After equilibrium was obtained (time zero), a 80 μl amino acid stock was added. The final dye concentration was 15 μM. All spectral curves were corrected for the effect of added 80 μl 15 mM Hepes/Tris alone (the medium of the solute stock solutions). The spectrophotometer was operated in the dual-wavelength mode with 580 and 610 nm (reference wavelength). Details of the individual experiments are given in the legends to the figures.

In the transport study of L-cysteine and L-cystine, major difficulties arises from the conversion of L-cysteine into L-cystine and vice versa. Therefore, all the experiments presented here concerning the uptake of L-cysteine were performed in the presence of dithiothreitol (DTT) to ensure that it was the transport of L-cysteine that was being studied instead of that of L-cystine. On the other hand, diamide (Azodicarboxylic acid bis[dimethylamide], Diazinedicarboxylic acid bis[N,N-dimethylamide]) was used as a stabilizing oxidant in experiments where the uptake of L-cystine was to be measured. We ascertained that addition of dithiothreitol or diamide (up to 10 mmol/liter) had no effect on sodium-dependent uptake of various amino acids or D-glucose (data not shown).

To determine the sodium/amino acid coupling ratio the "activation method" described by Turner and Moran (1982b) was used. In these experiments, as described in legends to figures, the vesicles were voltage-clamped by addition of valinomycin in order to minimize variations in the transmembrane electrical potential.

Calculations

The results of concentration dependence of transport rates were analyzed on the assumption of Michaelis-Menten kinetics. When the data of filtration experiments indicated uptake by more than one transport system, the results were analyzed according to the following equation

$$\text{Uptake} = \frac{V_{max1}[S]}{K_{m1} + [S]} + \frac{V_{max2}[S]}{K_{m2} + [S]}$$

where K_m represents the substrate concentration that gives half-maximal uptake, V_{max} denotes maximal uptake, and [S] indicates initial concentration of substrate. Subscripts 1 and 2 refer to the first and the second transport system, respectively. The calculations were performed by a computer-analyzed statistical iteration procedure (Jacobsen et al., 1982). The data from the stoichiometric studies were analyzed by the equation (Turner and Moran, 1982a):

$$\text{Flux} = V_{max}[A]^n/(K_{0.5}^n + [A]^n)$$

The equation assumes the existence of n essential cooperative site(s) for the activator A per amino acid site. According to this equation a plot of flux/$[A]^n$ against flux for the correct value of n will yield a straight line with slope $1/K_{0.5}^n$.

Results

Uptake of L-cysteine and L-cystine by luminal membrane vesicles from pars convoluta

Figure 1 describes the uptake of radioactive L-cysteine as a function of time by luminal membrane vesicles from the pars convoluta. In the presence of an inwardly directed Na^+-gradient at pH 7.5 (curve 3), the initial uptake of L-cysteine was rapid and reached a maximal value of approximately 2.8 nmol/mg protein within 45 s of incubation. Thereafter the intravesicular concentration of L-cysteine decreased, indicative of an increased efflux during dissipation of the Na^+-gradient, resulting in an equilibrium state after 60 min. At pH 5.5, in the presence or in the absence of an inwardly directed H^+-gradient, no significant increase in the Na^+-gradient-dependent accumulation of L-cysteine was observed (curve 1 and 2). Moreover, no overshoot was found for L-cysteine in the presence of a K^+-gradient or in the presence of a K^+- and H^+-gradient (curve 4 and 5).

Figure 2 depicts the time course of L-cystine uptake in the pars convoluta. An external Na^+-gradient led to an enhancement in the uptake of the amino acid (curve 1), compared with the uptake observed in the presence of a K^+-gradient (curve 3). Imposition of an H^+-gradient, in the presence (curve 2) or in the absence (curve 4) of a Na^+-gradient, did not result in an additional uptake of L-cystine. After 60 min there is a further increase in the uptake of

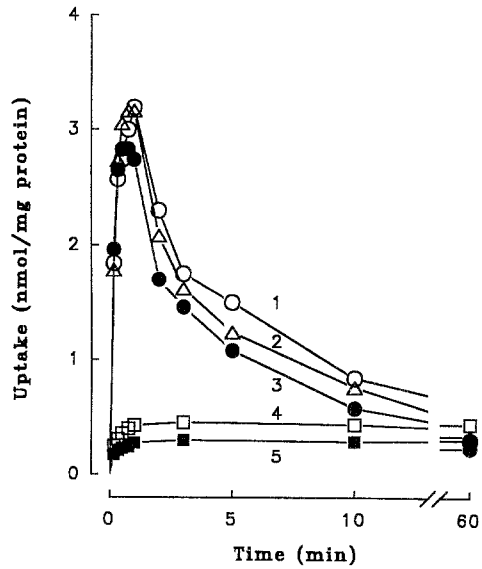

Fig. 1. Time course of L-cysteine uptake by luminal membrane vesicles from pars convoluta. 20 μl of vesicle suspension loaded with 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5, was incubated at different time intervals in 100 µl of incubation mixture consisting of 155 mM NaCl, 100 μM L-cysteine, 3 pM L-[^{35}S]-cysteine and 1 mM dithiothreitol in 15 mM Mes/Tris, pH 5.5 (curve 1) or in 15 mM Hepes/Tris, pH 7.5 (curve 3). In curves 4 and 5 NaCl in the incubation media was substituted with KCl at pH 7.5 and pH 5.5, respectively. In curve 2 the vesicles were loaded with 310 mM mannitol, 15 mM Mes/Tris, pH 5.5 and were incubated in 155 mM NaCl, 15 mM Mes/Tris pH 5.5. Results are the mean value of at least three experiments. S.D. was always less than 10%

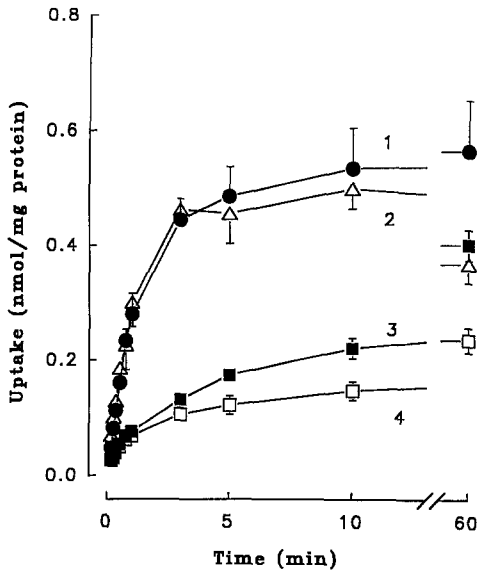

Fig. 2. Time course of L-cystine uptake by luminal membrane vesicles from pars convoluta. 20 μl of vesicle suspension loaded with 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5, was incubated at different time intervals in 100 μl of incubation mixture consisting of 155 mM NaCl, 25 µM L-cystine, 30 µM L-[^{35}S]-cystine and 3 mM diamide in 15 mM Hepes/Tris, pH 7.5 (curve 1), or in 15 mM Mes/Tris, pH 5.5 (curve 2). In curves 3 and 4 NaCl in the incubation media was substituted with KCl at pH 7.5 and pH 5.5, respectively. Results are given as mean values ± S.D. of at least three experiments

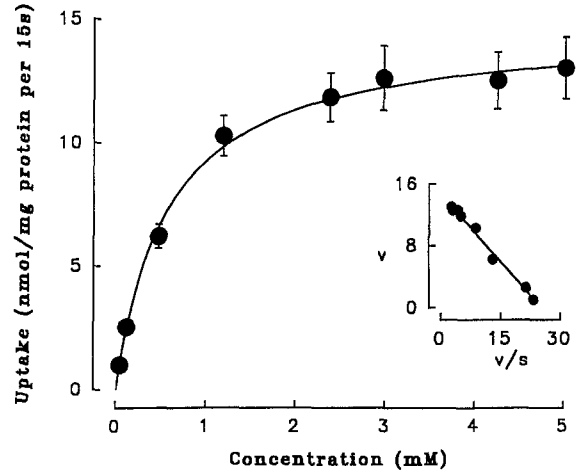

Fig. 3. Kinetics of L-cysteine uptake by luminal membrane vesicles from pars convoluta. 20 μl portion of vesicle suspension, loaded with 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5, was added to 100 μl of incubation medium containing 155 mM NaCl, 20 pM L-[^{35}S]-cysteine, 2.5 mM dithiothreitol, and various concentrations of unlabeled L-cysteine ranging from 0.035 to 5 mM (final concentration) in 15 mM Hepes/Tris buffer, pH 7.5. The values plotted have been corrected for non-saturable simple diffusion by subtracting the uptakes measured in KCl from the uptakes in NaCl. Inset shows the results in an Eadie-Hofstee plot. V represents the rate of transport at substrate concentration S. Results are given as mean values ± S.D. of three experiments

L-cystine in presence of KCl, a phenomenon which can be attributed to binding of amino acid by the membranes. By contrast the difference in the 60 min uptake values in the presence of various Na^+-gradients can not be explained at present.

Figure 3 shows the initial uptake of radioactive L-cysteine at increasing medium concentrations of the amino acid in the presence of a Na^+-gradient.

Table 1. Binding of L-cystine to luminal membrane vesicles from pars convoluta

Gradient	pH_{in}	pH_{out}	
		7.5	5.5
NaCl	7.5	0.25 ± 0.01	0.13 ± 0.01
NaCl	5.5	0.15 ± 0.01	0.08 ± 0.01
KCl	7.5	0.15 ± 0.04	0.09 ± 0.01
KCl	5.5	0.12 ± 0.01	0.08 ± 0.02

20 μl of vesicle suspension loaded with 310 mM mannitol was incubated in 100 μl of incubation mixture consisting of 155 mM NaCl or 155 mM KCl, 25 μM L-cystine, 30 μM L-[^{35}S]-cystine and 3 mM diamide. 15 mM Hepes/Tris (pH 7.5) or 15 mM Mes/Tris (pH 5.5) was used as buffer in all the solutions. 1 ml ice-cold trichloroacetic acid (11% w/v) was added to the samples after 10 min of incubation. The samples were placed in ice for 15 min before they were filtered and washed twice with 2 ml of trichloroacetic acid (5%). The results are given as nmol L-cystine bound per mg protein. The results are given as means ± S.D. of 3–5 experiments.

The values given have been corrected for non-saturable simple diffusion by subtracting the uptakes measured in KCl from the uptakes in NaCl. The linearity of the Eadie-Hofstee plot (inset) indicates the existence of a single transport system for the uptake of L-cysteine in the luminal membrane vesicles from pars convoluta. Computerized calculations resulted in the following kinetic parameters: K_m = 0.58 ± 0.05 mM and V_{max} = 14.63 ± 0.81 nmol/mg protein per 15 s.

Similar experiments were carried out with L-cystine. However, as also noted by Biber et al. (1983), no saturation-tendency was ever observed at L-cystine concentrations up to 0.4 mM. Therefore, due to the poor solubility of L-cystine, we have not been able to determine the number of transport systems involved in the reabsorption of this amino acid (results not shown). However, compared with the uptake of L-cysteine at similar low concentration Na^+-dependent influx rates are lower, and non-specific (Na^+-independent) uptake relatively higher. This can be attributed to binding of a significant fraction of L-cystine as also indicated by the progressive increase in non-specific (Na^+-independent) uptake during 60 min incubation. McNamara et al. (1981) have demonstrated that the amount of amino acid bound to vesicle membranes can be estimated after precipitation of L-cystine/protein complexes with trichloroacetic acid. Our results given in Table 1 show that the binding of L-cystine is more pronounced at pH 7.5 than at pH 5.5. The Table 1 also demonstrates the tendency for L-cystine to be bound in larger amounts in the presence of a Na^+-gradient than in the presence of a K^+-gradient. This is in accordance with Biber et al. (1983) who concluded that most of the binding sites are located on the interior side of the vesicles.

Uptake of L-cysteine and L-cystine by luminal membrane vesicles from pars recta

Figure 4 illustrates the time course of radioactive L-cysteine uptake by luminal membrane vesicles from the pars recta. As in the case of pars convoluta a transient accumulation of L-cysteine was seen in the presence of a NaCl gradient (curve 2), and a maximal level of approximately 2.4 nmol/mg protein was observed after 45 s of incubation. An equilibrium state was obtained within 60 min. However, the imposition of an H^+-gradient

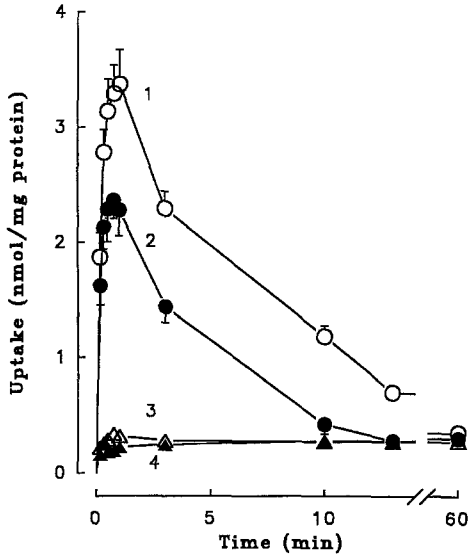

Fig. 4. Time course of L-cysteine uptake by luminal membrane vesicles from pars recta. 20 μl of vesicle suspension loaded with 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5, was incubated at different time intervals in 100 μl of incubation mixture consisting of 155 mM NaCl, 100 μM L-cysteine, 3 pM L[^{35}S]-cysteine and 1 mM dithiothreitol in 15 mM Mes/Tris, pH 5.5 (curve 1), or in 15 mM Hepes/Tris, pH 7.5 (curve 2). In curve 3 the vesicles were loaded with 155 mM NaCl, 15 mM Hepes/Tris, pH 7.5 and incubated in 155 mM NaCl, 15 mM Hepes/Tris pH 7.5. In curve 4 the vesicles were loaded with 310 mM mannitol, 15 mM Mes/Tris, pH 5.5 and incubated in 155 mM NaCl, 15 mM Mes/Tris pH 5.5. Results are given as mean values ± S.D. of at least three experiments

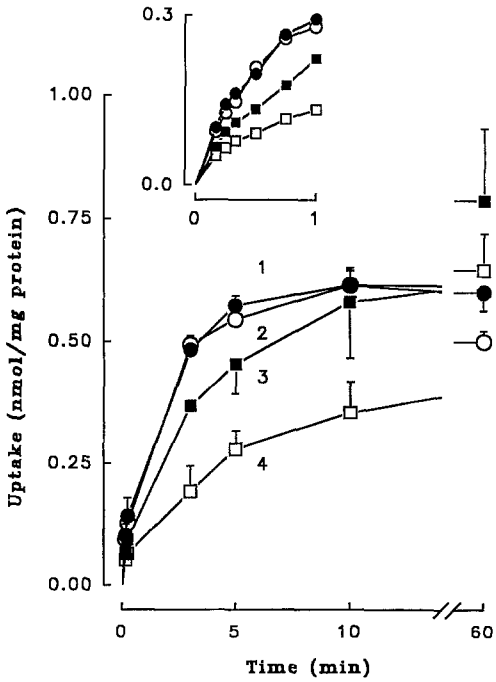

Fig. 5. Time course of L-cystine uptake by luminal membrane vesicles from pars recta. 20 μl of vesicle suspension loaded with 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5, was incubated at different time intervals in 100 μl of incubation mixture consisting of 155 mM NaCl, 25 μM L-cystine, 30 μM L-[^{35}S]-cystine and 3 mM diamide in 15 mM Hepes/Tris, pH 7.5 (curve 1), or 15 mM Mes/Tris, pH 5.5 (curve 2). In curves 3 and 4 NaCl in the incubation media was substituted with KCl at pH 7.5 and pH 5.5, respectively. The inset shows the initial uptake measured within the first minute of incubation. Results are given as mean values ± S.D. of at least three experiments

(extravesicular > intravesicular) in this case resulted in a significant increase in the Na^+-dependent uptake of L-cysteine (curve 1), but incubation at a lower pH without an H^+-gradient (i.e. $pH_{out} = pH_{in} = 5.5$) abolished both the H^+- and Na^+-dependent overshoot of L-cysteine (curve 3). Furthermore an inwardly directed H^+-gradient by itself did not exhibit an overshoot phenomenon even when Na^+ was present, but without a Na^+-gradient (curve 4). Nor did we find any transient accumulation of amino acid in the presence of a K^+-gradient or in the presence of both a K^+- and an H^+-gradient (data not shown).

Figure 5 shows time-dependent uptake of L-cystine in vesicles from the straight part of the proximal tubule. Curve 1 illustrates that an inwardly directed Na^+-gradient (i.e. $pH_{out} = pH_{in} = 7.5$) resulted in a slight, but significant stimulation of L-cystine transport compared with curve 3, where sodium is replaced with potassium. Imposition of an H^+-gradient markedly reduced the uptake of L-cystine in the presence of a K^+-gradient (curve 4), but a similar reduction in L-cystine uptake was not observed in the presence of a Na^+-gradient (curve 2). At $pH_{out} = pH_{in} = 5.5$, the uptake of L-cystine was decreased both in the presence of a Na^+-gradient or in the presence of a K^+-gradient (data not shown).

Figure 6 illustrates the sodium-dependent uptake of radioactive L-cysteine at increasing medium concentrations of the amino acid. The data have been corrected for passive diffusion by subtraction of the values obtained in the presence of equimolar concentration of KCl instead of NaCl. An Eadie-Hofstee analysis of the data is given in the inset, where the curvilinear plot

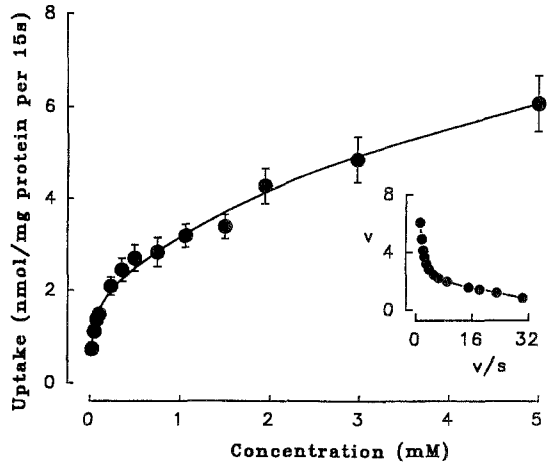

Fig. 6. Kinetics of L-cysteine uptake by luminal membrane vesicles from pars recta. 20 μl portion of vesicle suspension, loaded with 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5, was added to 100 μl of incubation medium containing 155 mM NaCl, 20 pM L-[^{35}S]-cysteine, 2.5 mM dithiothreitol, and various concentrations of unlabeled L-cysteine ranging from 0.014 to 5 mM (final concentration) in 15 mM Hepes/Tris buffer, pH 7.5. The values plotted have been corrected for non-saturable simple diffusion by subtracting the uptakes measured in KCl from the uptakes in NaCl. Inset shows the results in an Eadie-Hofstee plot. V represents the rate of transport at substrate concentration S. Results are given as mean values ± S.D. of three experiments

Table 2. Binding of L-cystine to luminal membrane vesicles from pars recta

Gradient	pH_{in}	pH_{out}	
		7.5	5.5
NaCl	7.5	0.37 ± 0.05	0.22 ± 0.12
NaCl	5.5	0.46 ± 0.03	0.28 ± 0.02
KCl	7.5	0.33 ± 0.04	0.17 ± 0.07
KCl	5.5	0.42 ± 0.02	0.29 ± 0.03

20 μl of vesicle suspension loaded with 310 mM mannitol was incubated in 100 μl of incubation mixture consisting of 155 mM NaCl or 155 mM KCl, 25 μM L-cystine, 30 μM L-[^{35}S]-cystine and 3 mM diamide. 15 mM Hepes/Tris (pH 7.5) or 15 mM Mes/Tris (pH 5.5) was used as buffer in all the solutions. 1 ml ice-cold trichloroacetic acid (11% w/v) was added to the samples after 10 min of incubation. The samples were placed in ice for 15 min before they were filtered and washed twice with 2 ml of trichloroacetic acid (5%). The results are given as nmol L-cystine bound per mg protein. The results are given as means ± S.D. of 3–5 experiments.

indicates the existence of multiple transport components for the uptake of L-cysteine in pars recta. Computer analysis assuming two transport systems resulted in following kinetic values: K_{m1} = 0.03 ± 0.01 mM, V_{max1} = 1.88 ± 0.20 nmol/15 s per mg protein, K_{m2} = 5.84 ± 0.53 mM and V_{max2} = 9.06 ± 0.99 nmol/15 s per mg protein. The same experiments were done for L-cystine in luminal membrane vesicles from pars recta. However the influx of L-cystine never tended towards a saturation level, making it impossible to determine the kinetic parameters (data not shown). Since it is known that L-cystine binds to the membrane vesicles, a series of experiments were performed to quantify the amount of amino acid binding in pars recta (Table 2). The results demonstrated a marked decrease in binding of L-cystine by a change in the extravesicular pH from 7.5 to 5.5.

Stoichiometric studies of Na^+ cotransport with L-cysteine and L-cystine in the proximal tubule

Experiments with increasing sodium concentrations were performed in order to evaluate the coupling ratio of sodium:amino acid in the two luminal segments of the nephron. This was done by use of the "activation method" (Turner and Moran, 1982a), with voltage-clamped membrane vesicles and an incubation time of 15 s. Results from a representative experiment for L-cysteine and L-cystine in luminal membrane vesicles from pars convoluta are given in Fig. 7. It is apparent from Fig. 7A that the uptake values of L-cysteine versus sodium concentration exhibited a Michaelis Menten type dependency suggesting a 1:1 stoichiometry. This is confirmed in Fig. 7B where n = 1 results in a straight line relationship. Similar results were ob-

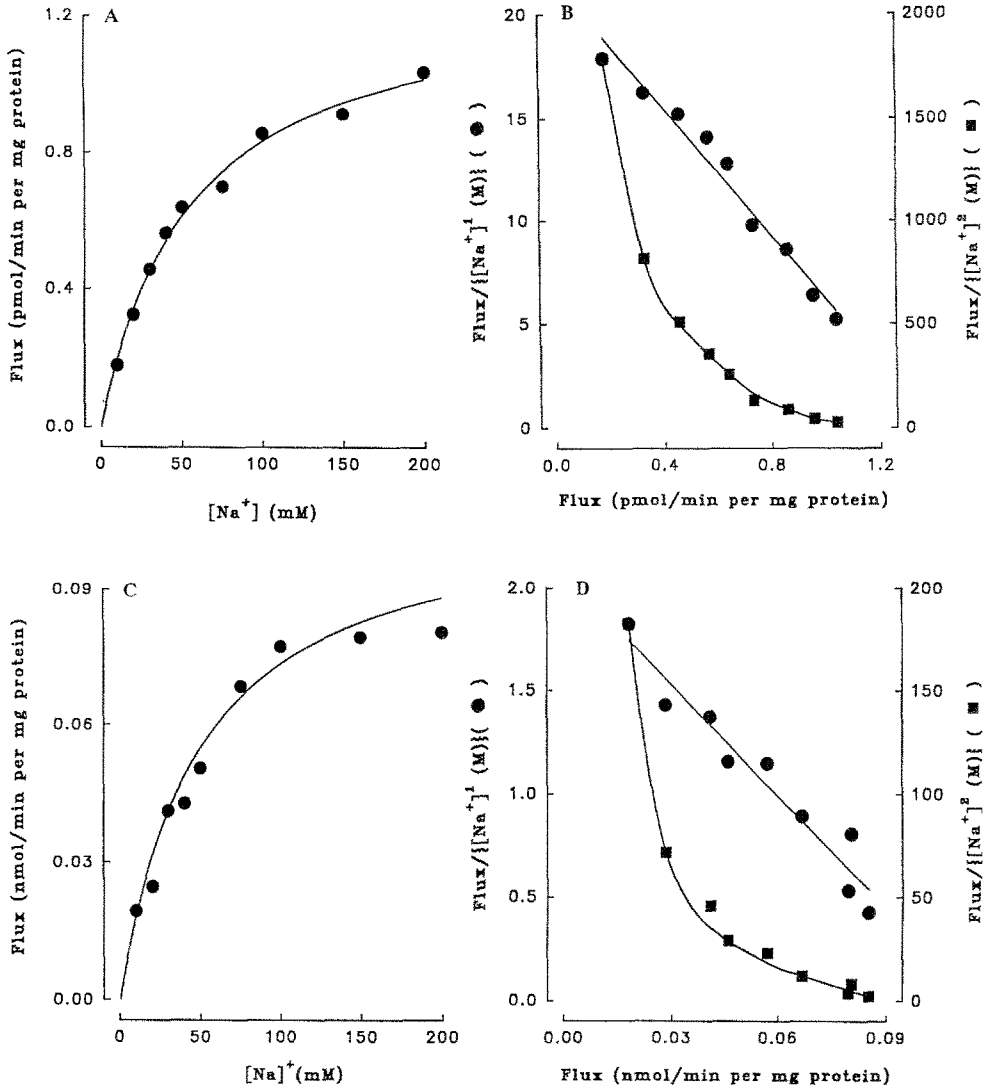

Fig. 7. Effect of Na^+ concentration on the uptake of L-cysteine and L-cystine by luminal membrane vesicles from pars convoluta. Vesicles were pre-incubated in 15 mM Hepes/Tris buffer, pH 7.5, containing 100 mM KSCN, 700 mM mannitol and valinomycin at a concentration of 12.5 μg/mg protein. The incubation media contained 15 mM Hepes/Tris, pH 7.5, 100 mM KSCN, 300 mM mannitol, 6 pM L-[^{35}S]-cysteine and 20 nM L-cysteine, 2.5 mM dithiothreitol and various concentrations of NaCl ranging from 0–200 mM (final concentration). Choline chloride was used to replace sodium chloride isosmotically to obtain various sodium concentrations studied. The composition of the stop buffer was 15 mM Hepes/Tris (pH 7.5), 400 mM mannitol and 300 mM NaCl. The Na^+ ion-gradient-driven uptake values plotted have been corrected for a passive diffusion component measured as the uptake of L-cysteine in the absence of a Na^+ gradient. **A** Plot of flux vs sodium concentration. **B** Plots of flux/$[Na^+]^n$ vs flux for n = 1 and n = 2. The same experiments were done for L-cystine (40 μM L-cystine, 20 μM L-[^{35}S]-cystine and 5 mM diamide). **C** Plot of flux vs sodium concentration. **D** Plots of flux/$[Na^+]^n$ vs flux for n = 1 and n = 2. The results shown are from a representative experiment (n = 5)

tained for L-cystine as illustrated in Fig. 7C and 7D. The same kind of experiments were carried out for L-cysteine and L-cystine in the straight part of the proximal tubule (data not shown). We conclude that there is a 1:1 stoichiometry for all the Na^+-dependent transport components involved in the uptake of L-cysteine or L-cystine in the proximal tubule. Since the "activation method" does not distinguish between catalytic or energetic coupling, the uptake of L-cysteine and L-cystine was also studied by spectrophotometry, with the potential-sensitive dye 3'3-diethyloxadicarbocyanine (Kragh-Hansen et al., 1982). It is seen from Fig. 8A that addition of L-cysteine to membrane vesicles from pars convoluta in the presence of an extravesicular > intravesicular Na^+-gradient resulted in a marked depolarization (curve 1), whereas no effect was ever observed in the presence of a K^+-gradient (curve 2). These findings suggest an electrogenic uptake mechanism for L-cysteine caused by a direct coupling of amino acid flux with sodium flux. By contrast, no absorbance changes were found for L-cystine in the presence of a Na^+- or K^+-gradient in vesicles from the convoluted part of the proximal tubule (curve 3 and 4). A possible explanation may be the existence of a catalytic coupling between the amino acid and the sodium ion. On the other hand, we can not exclude a co-transport mechanism involving the influx of 1 Na^+, 1 L-cystine and 1 anion. In vesicles from pars recta (Fig. 8B), addition of both L-cysteine or L-cystine to dye-membrane-vesicles suspensions revealed an electrogenic transport process in the presence of an

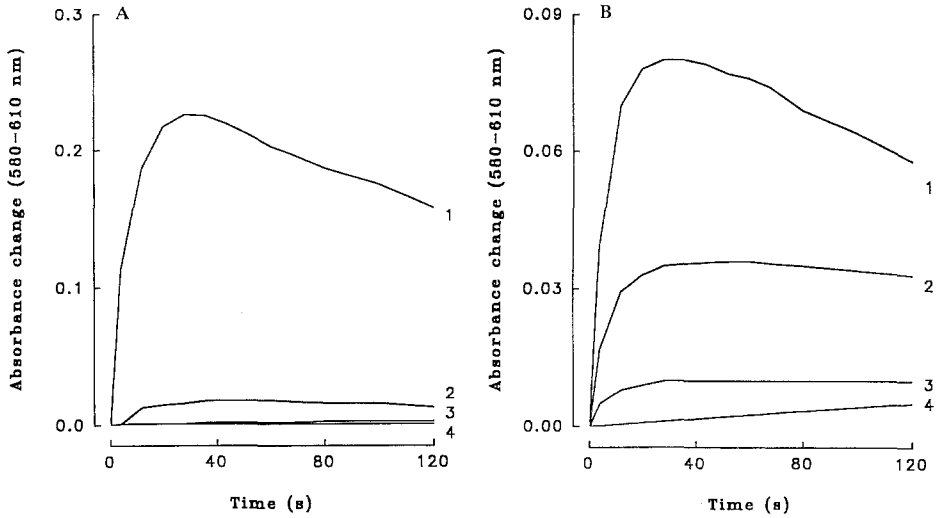

Fig. 8. Uptake of L-cysteine and L-cystine by luminal membrane vesicles form pars convoluta (A) and from pars recta (B) as registered by the potential-sensitive dye 3,3-diethyloxacarbocyanine iodide. **A** Uptake of 10 mM L-cysteine, 5 mM dithiothreitol in the presence of a NaCl-gradient (curve 1) or a KCl-gradient (curve 2) and uptake of 4.4 mM L-cystine, 5 mM diamide in the presence of a NaCl-gradient (curve 3) or a KCl-gradient (curve 4) in membrane vesicles from pars convoluta. **B** Uptake of 10 mM L-cysteine, 5 mM dithiothreitol in the presence of a NaCl-gradient (curve 1) or a KCl-gradient (curve 4) and uptake of 4.4 mM L-cystine, 5 mM diamide in the presence of a NaCl-gradient (curve 2) or a KCl-gradient (curve 3) in membrane vesicles from pars recta. The results are from a representative experiment (n = 3)

inwardly directed Na^+-gradient, in accordance with a net transfer of sodium ions across the membrane for both amino acids.

Competition experiments for L-cysteine and L-cystine in the proximal tubule

Table 3 illustrates the effect of different amino acids on the sodium-dependent transport of L-cysteine and L-cystine in luminal membrane vesicles from pars convoluta. In these experiments D-glucose was used as a control of non-specific inhibition caused by sodium depletion. It is seen that addition of low or high concentrations of basic or acidic amino acids had no effect on the uptake of L-cysteine, whereas all the neutral amino acids, with the exception of L-proline at low concentration, inhibited the influx of L-cysteine.

Basic amino acids and most neutral amino acids had an unequivocal inhibition effect on the influx of L-cystine. But surprisingly, some of the neutral amino acids while inhibiting at 5 mM, at 0.5 mM apparently either stimulated or had no effect on L-cystine influx. This was the case for L-phenylalanine,

Table 3. Effect of different amino acids on the sodium-dependent transport of L-cysteine and L-cystine in luminal membrane vesicles from pars convoluta

Inhibitor concentration (mM)	L-cystine		L-cysteine	
	0.5	5.0	0.5	5.0
D-Glucose	0.90 ± 0.12	0.73 ± 0.12	0.91 ± 0.07	0.80 ± 0.08
L-Alanine	0.63 ± 0.10	0.34 ± 0.02	0.60 ± 0.05	0.25 ± 0.07
L-Phenylalanine	1.30 ± 0.04	0.43 ± 0.02	0.37 ± 0.05	0.10 ± 0.01
L-Valine	0.71 ± 0.07	0.39 ± 0.10	0.46 ± 0.10	0.19 ± 0.08
L-Serine	0.77 ± 0.10	0.36 ± 0.10	0.62 ± 0.06	0.25 ± 0.07
L-Leucine	1.07 ± 0.07	0.31 ± 0.03	0.45 ± 0.09	0.13 ± 0.09
L-Isoleucine	0.50 ± 0.04	0.33 ± 0.10	0.43 ± 0.08	0.13 ± 0.06
L-Proline	1.26 ± 0.13	0.95 ± 0.04	0.87 ± 0.04	0.64 ± 0.05
L-Glycine	0.94 ± 0.10	0.70 ± 0.08	0.77 ± 0.05	0.57 ± 0.09
L-Arginine	0.36 ± 0.16	0.22 ± 0.07	0.96 ± 0.07	0.89 ± 0.07
L-Lysine	0.54 ± 0.14	0.25 ± 0.07	0.86 ± 0.08	0.89 ± 0.09
L-Glutamate	1.19 ± 0.07	1.00 ± 0.08	1.09 ± 0.03	0.87 ± 0.06
L-Methionine	1.18 ± 0.33	0.53 ± 0.12	0.36 ± 0.09	0.07 ± 0.08
L-Tryptophan	0.76 ± 0.17	–	0.50 ± 0.06	0.12 ± 0.01
L-Threonine	0.78 ± 0.15	0.48 ± 0.10	0.58 ± 0.10	0.25 ± 0.05
L-Asparagine	0.96 ± 0.08	0.45 ± 0.05	0.58 ± 0.09	0.25 ± 0.08
L-Glutamine	0.96 ± 0.09	0.43 ± 0.09	0.58 ± 0.03	0.23 ± 0.07
L-Ornithine	0.35 ± 0.10	0.17 ± 0.10	1.01 ± 0.04	0.87 ± 0.09

Vesicles were loaded in 310 mM mannitol, 15 mM Hepes/Tris (pH 7.5), and incubation medium contained 155 mM NaCl, 15 mM Hepes/Tris (pH 7.5), and different concentrations of the examined amino acid or glucose as indicated in the Table. The concentration of L-cystine and L-cysteine was 40 μM L-[^{35}S]-cystine in 5.5 mM diamide and 5 nM L-[^{35}S]-cysteine in 5 mM dithiothreitol, respectively. Uptake in the absence of the test compound is designated as 1.00. Incubation time was 15 s. Uptake values have been corrected for a passive diffusion compound. The results are given as means ± S.D. of 3–5 experiments.

L-leucine, L-asparagine, L-glutamine and L-methionine. There was no decrease in the uptake of L-cystine in the presence of L-proline or the acidic amino acid L-glutamate.

The results from competition experiments in vesicles from pars recta are given in Table 4. Since it is well known that the coupling ratio of D-glucose is 1 sugar:2 Na^+ in the straight part of the proximal tubule only half the amount of D-glucose was used as compared to the amino acids tested. The influx of L-cysteine was slightly inhibited by the basic amino acids L-lysine and L-arginine, but no effect was observed with L-ornithine and the acidic amino acid L-glutamate. The uptake of L-cysteine in pars recta is reduced by L-proline at both concentrations tested, whereas L-tryptophan only inhibited the influx of L-cysteine at high inhibitor concentration. As to L-cystine, reduction in the uptake of amino acid was only observed at high concentration for L-asparagine, L-glutamine or L-phenylalanine, while the remaining neutral and basic amino acids tested decreased the influx of L-cystine at both concentrations. No effect was ever observed with L-glutamate.

Table 4. Effect of different amino acids on the sodium-dependent transport of L-cysteine and L-cystine in luminal membrane vesicles from pars recta

Inhibitor concentration (mM)	L-cystine		L-cysteine	
	0.5	5.0	0.5	5.0
D-Glucose	0.98 ± 0.05*	0.94 ± 0.12**	0.93 ± 0.08*	0.78 ± 0.09**
L-Alanine	0.59 ± 0.02	0.33 ± 0.10	0.33 ± 0.04	0.02 ± 0.04
L-Phenylalanine	0.80 ± 0.14	0.37 ± 0.12	0.39 ± 0.08	0.16 ± 0.09
L-Valine	0.50 ± 0.10	0.30 ± 0.02	0.23 ± 0.10	0.00 ± 0.00
L-Serine	0.62 ± 0.10	0.31 ± 0.02	0.24 ± 0.05	0.00 ± 0.00
L-Leucine	0.59 ± 0.04	0.22 ± 0.04	0.31 ± 0.01	0.00 ± 0.00
L-Isoleucine	0.43 ± 0.10	0.31 ± 0.01	0.37 ± 0.04	0.10 ± 0.06
L-Proline	0.76 ± 0.07	0.65 ± 0.16	0.52 ± 0.06	0.25 ± 0.07
L-Glycine	0.67 ± 0.03	0.39 ± 0.02	0.55 ± 0.03	0.08 ± 0.09
L-Arginine	0.44 ± 0.08	0.31 ± 0.05	0.74 ± 0.07	0.75 ± 0.01
L-Lysine	0.56 ± 0.14	0.26 ± 0.12	0.64 ± 0.11	0.66 ± 0.02
L-Glutamate	1.02 ± 0.15	0.85 ± 0.10	0.83 ± 0.07	0.70 ± 0.08
L-Methionine	0.78 ± 0.05	0.40 ± 0.08	0.11 ± 0.09	0.02 ± 0.04
L-Tryptophan	0.79 ± 0.12	–	0.85 ± 0.12	0.31 ± 0.05
L-Threonine	0.74 ± 0.05	0.37 ± 0.08	0.22 ± 0.09	0.06 ± 0.10
L-Asparagine	0.67 ± 0.21	0.32 ± 0.20	0.54 ± 0.10	0.00 ± 0.00
L-Glutamine	0.72 ± 0.22	0.41 ± 0.12	0.33 ± 0.01	0.17 ± 0.03
L-Ornithine	0.40 ± 0.03	0.20 ± 0.03	0.92 ± 0.12	0.89 ± 0.01

Vesicles were loaded in 310 mM mannitol, 15 mM Hepes/Tris (pH 7.5), and incubation medium contained 155 mM NaCl, 15 mM Hepes/Tris (pH 7.5), and different concentrations of the examined amino acid or glucose as indicated in the Table. The concentration of L-cystine and L-cysteine was 40 μM L-[^{35}S]-cystine in 5.5 mM diamide and 5 nM L-[^{35}S]-cysteine in 5 mM dithiothreitol, respectively. Uptake in the absence of the test compound is designated as 1.00. Incubation time was 15 s. Uptake values have been corrected for a passive diffusion compound. The results are given as means ± S.D. of 3–5 experiments.

* D-Glucose concentration was 0.25 mM.

** D-Glucose concentration was 2.50 mM.

Discussion

In spite of numerous reports the complicated renal transport mechanism for L-cysteine and L-cystine still needs to be clarified. The purpose of the present study was to characterize the uptake of the above-mentioned amino acids with luminal membrane vesicles prepared from the pars convoluta and the pars recta of rabbit proximal tubule. These preparations are well established for study of transport of amino acids and other organic anions. Our examination demonstrated that the influx of L-cysteine is a sodium-dependent process in both segments of the nephron. This is in accordance with previous reports (Samarzija and Frömter, 1982a; Stieger et al., 1983), where microperfusion techniques and membrane vesicles from rat whole cortex were used. Imposition of an H^+-gradient only enhanced the uptake of L-cysteine in vesicles from pars recta, and only in the concomitant presence of a Na^+-gradient. Thus, an inwardly directed Na^+-gradient at pH 5.5 without an H^+-gradient drastically reduced the sodium-gradient-dependent influx of the amino acid when compared with the uptake at pH 7.5.

In regard to L-cystine, we found that although the presence of a Na^+-gradient (extravesicular > intravesicular) stimulated the influx of L-cystine in both the convoluted and the straight part of the tubule, this process took place without the production of an overshoot. Similar results have been obtained by other investigators (McNamara et al., 1981; Biber et al., 1983) from preparations of rat whole cortex. Although the initial influx of L-cystine in the presence of a K^+-gradient was lower than in a Na^+-gradient the uptake of amino acid rose and approached that observed in Na^+ after long incubation periods. This phenomenon presumably reflects that a considerable amount of amino acid is bound to the membranes. Quantification of L-cystine bound to the vesicles from pars convoluta showed that the amount of involved amino acid is greater when the extravesicular pH was 7.5 compared with 5.5. This tendency was not altered when intravesicular pH was reduced from 7.5 to 5.5. In an earlier study, Biber et al. (1983) concluded that most of the binding sites for L-cystine are located to the interior side of the vesicles. This is supported by the fact that our results in Table 1 indicate a higher degree of binding in the presence a Na^+-gradient than in the presence of a K^+-gradient. Thus, taken together this may suggest that L-cystine molecules are partly bound to the inside but also partly bound to the outside of the membrane vesicles. In pars recta, the binding of L-cystine as a function of extravesicular pH exhibited the same pattern as in pars convoluta, except that the degree of binding was higher. Moreover, our results did not show any noticeable difference in the amount of amino acid bound to the membranes when sodium in the incubation media was replaced by potassium in this part of the nephron.

We also examined the effect of an inwardly directed H^+-gradient on the transport of L-cystine. To do so, we had to look at the initial uptake values of the amino acid, where the contribution from membrane bound L-cystine was relatively small. We found that a proton-gradient did not result in any additional uptake of amino acid in the presence or in the absence of a sodium-gradient. This applied to pars convoluta as well as to pars recta of the nephron. These findings confirm data from rat whole cortex recently pub-

lished by Reynolds et al. (1991). On the other hand, if one looks at the uptake values obtained at 10 min after they have been corrected for the binding components given in Tables 1 and 2, our data may indicate that, at least in pars recta, the imposition of an H^+-gradient to a Na^+- or K^+-gradient results in the same uptake pattern as seen for L-cysteine. Thus, further studies are needed to improve our knowledge of the effect of a pH-gradient on the uptake of L-cystine in the proximal tubule.

Kinetic analysis of Na^+-dependent L-cysteine uptake revealed the existence of a single transport system (K_m = 0.58 ± 0.05 mM, V_{max} = 14.6 ± 0.81 nmol/15 s per mg protein) in vesicles from pars convoluta. By contrast, non-linear regression indicated the presence of more than one transport component in vesicles from pars recta (K_{m1} = 0.03 ± 0.01 mM, V_{max1} = 1.88 ± 0.20 nmol/15 s per mg protein, K_{m2} = 5.84 ± 0.53 mM and V_{max2} = 9.06 ± 0.99 nmol/15 s per mg protein). Samarzija et al. (1982a) have previously provided evidence for a single transport system for L-cystine by microperfusion studies on rat kidney. On the other hand, Stieger et al. (1993) reported the presence of both a high and a low-affinity transport component in luminal membrane vesicles from rat whole cortex. These conflicting reports emphasize the importance of performing segment-specific examinations of the transport systems located in the proximal tubule. A number of other neutral amino acids exhibit the same distribution of Na^+-dependent transport components along this part of the nephron, namely L-alanine, glycine, L-leucine, L-isoleucine and L-valine (Jørgensen and Sheikh, 1987; Røigaard-Petersen et al., 1990; Jørgensen et al., 1990). Our examination of the Na^+-dependent uptake of L-cystine as a function of L-cystine concentration in both parts of the tubule did not result in any kinetic parameters because of the lack of saturation. This is in good agreement with a previous report dealing with membrane vesicles from rat whole cortex (Biber et al., 1983). On the other hand, other communications based on the same kind of rat vesicles have described one or two transport components for the amino acid L-cystine (McNamara et al., 1981; States et al., 1987).

Stoichiometric studies using the 'activation method' indicated a coupling ratio of 1 Na^+:1 amino acid for the transport systems involved in the uptake of L-cysteine and L-cystine along the proximal tubule. Furthermore, we found by spectrophotometric experiments that, apart from the influx of L-cystine in pars convoluta, the transport of the two amino acids across the membranes is an electrogenic process, suggesting a net transfer of sodium ions. A possible explanation concerning the non-electrogenic influx of L-cystine in the convoluted part of the tubule has previously been discussed in this paper.

Examination of the effect of different amino acids on the uptake of L-cysteine indicated that acidic or basic amino acids did not influence the influx of L-cysteine in pars convoluta. This is in good agreement with previous reports (Völkl and Silbernagl, 1982b; Stieger et al., 1983; Samarzija and Frömter, 1982b). However, as already mentioned L-lysine and L-arginine had a slight inhibitory effect on uptake of L-cysteine in pars recta. Stieger et al. (1983) concluded that the transport of L-cysteine is inhibited by neutral amino acids in the proximal tubule. This conclusion was based on the inhibitory

effect of L-alanine and L-phenylalanine on L-cysteine uptake in membrane vesicles from rat whole cortex. The data presented in this study support these observations. Competition experiments for L-cystine showed, as also reported by other investigators (Dent and Rose, 1951; Biber et al., 1983; Segal et al., 1977), that L-ornithine, L-arginine, and L-lysine reduced the uptake of L-cystine. Our study revealed that this phenomenon occurred in both segments of the proximal tubule. The resemblance of the structures of these amino acids especially the two amino groups seems to fit very well with a common transport system. By contrast to previous reports (McNamara et al., 1981; Segal et al., 1977), our experiments demonstrated that all the neutral amino acids tested, with the exception of L-proline in pars convoluta, inhibited the uptake of L-cystine even though some of the amino acids only reduced the influx of L-cystine at high inhibitor concentration, suggesting the presence of a common transport component but with different affinities.

On the assumption that rabbit kidneys are comparable with human kidneys, our results strongly suggest that L-cystine transport is shared by not only the basic amino acids but also by neutral amino acids. Due to the separate transport systems of basic and neutral amino acids (Samarzija and Frömter, 1982b), the transport of L-cystine must appear by two different systems. Consequently a defect in the common transport system with the basic amino acids can explain the phenomenon of classical cystinuria, which is characterized by hyperexcretion of L-cystine, lysine, arginine and ornithine in the urine. On the other hand the data presented in this paper do not provide us with a possible explication of the isolated type of cystinuria. However it should be pointed out that isolated type of cystinuria has only been observed in one patient (Brodehl et al., 1967).

Acknowledgement

This study was supported in part by the Danish Medical Research Council, Aarhus University Research Foundation, the Danish Foundation for the Advancement of Medical Science, The Novo Nordisk Foundation, The Michaelsen Foundation, and The Carlsberg Foundation.

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Received May 5, 1994