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Transport of L-Cysteine by Rat Renal Brush Border Membrane Vesicles

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Summary. Brush border membranes were isolated from rat renal cortex by a divalent cation precipitation method. L-^{35}S-cysteine uptake into the vesicles was measured by a rapid filtration method. Only minimal binding of the amino acid to the vesicles was observed. Sodium stimulates L-cysteine uptake specifically. Anion replacement experiments, experiments in the presence of potassium/valinomycin-induced diffusion potential as well as experiments with a potential-sensitive fluorescent dye document an electrogenic sodium-dependent uptake mechanism for L-cysteine. Tracer replacement experiments as well as the fluorescence experiments indicate a preferential transport of L-cysteine. Transport of L-cysteine is inhibited by L-alanine and L-phenylalanine but not by L-glutamic acid and the L-basic amino acids. Initial, linear influx kinetics provide evidence for the existence of two transport sites. The results suggest (a) sodium-dependent mechanism(s) for L-cysteine shared by other neutral amino acids.

Key Words L-cysteine transport · renal proximal tubule · brush border membrane vesicles · cotransport

Introduction

Renal proximal tubular amino acid transport has been characterized by different techniques (Sacktor, 1977; Frömter, 1979; Ullrich, 1979; Murer & Kinne, 1980; Schafer & Barfuss, 1980; Weiss, McNamara & Segal, 1981; Sibernagl, 1981; Mircheff et al., 1982). Experiments performed with intact tubules *in vivo,* with isolated perfused tubules *in vitro* or with isolated vesicles showed that amino acid reabsorption in the renal proximal tubule is sodium-dependent and secondary active. The concentrative mechanisms are located in the brush border membrane and represent sodium solute cotransport mechanisms.

Studies with brush border membrane vesicles provide an excellent model for an analysis of the sodium-amino acid cotransport mechanisms. In these studies the experimental conditions can be strictly controlled, and influences of the metabolism or contributions of the transport phenomena located at the opposite cell border can be excluded. For renal transport of L-cysteine/cystine, major difficulties arise from the conversion of L-cysteine into L-cystine and vice versa. In this respect, studies with vesicles offer the advantage of including oxidants or reductants into the experimental solutions in order to control the presence of cysteine and cystine, respectively. These maneuvers might cause unspecific alterations in the intact tubular system. Nevertheless, microperfusion experiments have been performed in the presence of such drugs. They indicated that L-cysteine transport is separated from L-cystine transport (Völkl & Silbernagl, 1982*a, b*). Electrophysiological experiments documented that L-cysteine transport across the rat renal proximal tubular brush border membrane is sodium-dependent and carries a positive charge (Samarzija & Frömter, 1982).

In vivo experiments on the transport of amino acids provided evidence for at least six different transport mechanisms (Frömter, 1979; Ullrich, 1979; Silbernagl, 1981). According to recent studies with brush border membrane vesicles isolated from rabbit proximal tubule, the multiplicity of transport mechanisms for amino acids seems to be even higher. Cross inhibition experiments led to a delineation of six different sodium-stimulated pathways only for neutral amino acids. Unfortunately, no data on L-cysteine transport were included in this report (Mircheff et al., 1982). In the past few years several papers on the transport of L-cysteine or L-cystine into renal brush border vesicles have been published. Surprisingly, the experimental conditions used in these studies did not distinguish clearly between L-cysteine transport and L-cystine transport, respectively (Segal, McNamara & Pepe, 1977; Busse, 1978; Hilden & Sacktor, 1981; McNamara, Pepe & Segal, 1981). Thus, the results presented might reflect a mixture of L-cysteine and L-cystine transport. In prelimi-

nary experiments on L-cysteine transport in rabbit renal brush border vesicles in the presence of dithiothreitol, Busse, Pohl and Bartel (1981) found evidence for a sodium independent transport mechanism. This is in clear contradiction to electrophysiological experiments (Frömter, 1979; Samarzija & Frömter, 1982).

In the present paper we are describing experiments on the transport of L-cysteine with rat renal brush border vesicles. In order to keep the substrate in its reduced form, dithiothreitol was included in all incubation media. L-cysteine transport was found to be sodium-dependent, stereospecific and to interact with the transport of the neutral amino acids L-alanine and L-phenylalanine. Evidence for the operation of two transport mechanisms was found.

Materials and Methods

Brush border membranes were prepared from male Wistar rats (190 to 250 g) by an EGTA/magnesium precipitation method, as described in detail elsewhere (Biber et al., 1981 *a*). The purity of the brush border membranes was routinely checked by measuring the enrichment of alkaline phosphatase and aminopeptidase M – two marker enzymes for the brush border membrane. The enrichment was 14- to 17-fold with respect to the starting homogenate. After the purification of the brush border membranes the vesicles were loaded with buffers as indicated in the figure legends and Tables. Usually the final pellet was suspended in 30 ml of desired buffer and rehomogenized with a glass-Teflon homogenizer. The membranes were collected by centrifugation and resuspended in the desired volume of final buffer by sucking approximately 30 times through a fine needle. Then the membranes were equilibrated for 1 hr at room temperature. For all membrane preparations sodium gradient-dependent D-glucose uptake was measured as a control. After 20 sec of incubation, D-glucose uptake was usually between four- and sixfold that observed after 90 min of incubation. The uptake of labeled L-^{35}S-cysteine was started by adding an aliquot of membrane vesicle suspension (10 to 20 µl) to the incubation medium (40 to 120 µl). It was terminated by the removal of an aliquot of the incubation mixture followed by immediate dilution into ice-cold stop solution and subsequent rapid filtration through Sartorius filters (cellulose nitrate, pore size 0.6 µm) kept under suction. All incubations were carried out at 25° C. For the rapid uptake measurements (initial uptake, kinetics) a semiautomatic set-up was used as described by Kessler, Tannenbaum and Semenza (1978) and now constructed by Innovativ AG (Adliswil, Switzerland). With this apparatus it was only possible to perform incubations at room temperature (21 to 23° C). Stop solution injected after the chosen incubation time was kept at 0° C. The composition of the incubation media is given in the Figure legends. The stop solutions were of similar composition as the sodium-containing incubation mixtures (approx. 20% hypertonic made by the addition of NaCl) but without L-cysteine. Using a specially designed funnel the diluted ice-cold membrane suspension could be filtered within 5 to 10 sec. Filters were washed with 3 ml of ice-cold stop solution. The radioactivity retained on the membrane filters was counted by liquid scintillation techniques. Cysteine uptake was calculated on the basis of the specific activity determined in each experiment. Unspecific binding of the isotopes was determined by adding the labeled substrates to the stop solution containing the vesicles at 0° C and by subsequent filtration. The unspecific binding was always less than 10% of the equilibrium uptake reached after 90 min of incubation of the vesicles at 25° C. Single experiments will be presented throughout the paper. All experiments were repeated at least three times with qualitatively identical results. Within the same experiment all uptake measurements were performed in triplicate or quadruplicate. The experimental scatter among the individual values was around 5%. For reasons not known to us, the absolute uptake values (pmol/mg protein) varied by a factor of about 1.5 from membrane preparation to membrane preparation. A possible explanation for this variation could be the animal variability and small differences in tissue preparation. As rat renal brush border membrane vesicles show a significant loss of transport activity by storing in liquid nitrogen, we decided not to use a large pool of frozen membranes for the various experiments. By this maneuver inter-animal and inter-experimental variations could be reduced (e.g. Stevens, Ross & Wright, 1982). As the conclusions of the present paper are always based on findings obtained within the same experiments the experimental scatter does not represent a major problem in evaluating the data obtained in this study. The kinetic parameters were calculated according to Spears, Sneyd and Loten (1971) with least-square regression and iterative stripping of data plotted according to Hanes-Woolf (S/v versus S). The calculations were performed with a HP 9815 calculator.

Protein was determined by using the BioRad protein assay kit using protein standard I. Alkaline phosphatase activity was assayed as described by Berner, Kinne and Murer (1976). Aminopeptidase M was analyzed as described by Haase et al. (1978).

For fluorescence measurements 20 µl of membrane vesicle suspension were added to 2 ml of incubation medium containing 3 µmol/liter 3,3′diethylthiadicarbocyanine iodide as a potential sensitive dye (Burckhardt & Murer, 1981). Fluorescence measurements were performed with a Shimadzu spectrofluorometer (RF 510, Tokyo, Japan) equipped with a red sensitive photomultiplier (R 446 U). The excitation was set to 622 nm (slit 5 nm) and the emission was recorded at 669 nm (slit 10 nm).

L-^{35}S-cysteine was purchased from NEN (Dreieich, FRG). 3,3′diethylthiadicarbocyanine iodide was from Eastman Kodak (Rochester, N.Y.). All other chemicals were at least analytical grade and were obtained from Merck (Darmstadt, FRG), Serva (Heidelberg, FRG) and Sigma (Munich, FRG).

Abbreviations

HEPES: N-2-hydroxethylpiperazine-N′-ethane sulfonic acid; Tris: Tris(hydroxymethyl)aminomethane; EGTA: Ethyleneglycol-bis-(β-amino-ethyl-ether)-N,N′-tetraacetic acid.

Results

As cysteine is easily oxidized into cystine, all the experiments to be presented were performed in the presence of dithiothreitol to make sure that the transport of cysteine was studied and not that of cystine. Such precautions have to be taken, as preliminary experiments have indicated that transport of cystine – experiments performed in the presence of oxidating agents – occur via an entirely different pathway (Biber et al., 1981 *b*). Experiments with

vesicles offer an advantage in this respect, as reducing or oxidizing conditions can be introduced without causing uncontrolled unspecific effects; e.g., dithiothreitol can be added up to concentration of 10 mmol/liter without significantly altering sodium-dependent D-glucose uptake and without reducing the vesicular volume (*data not shown; see also* Fig. 4*a*, 3).

General Properties

The influence of sodium on L-cysteine uptake into rat renal brush border membrane vesicles is shown in Fig. 1. L-cysteine uptake is strongly stimulated by a sodium gradient as compared to a potassium gradient, and a transient accumulation is observed in the presence of an inwardly directed sodium gradient. As will be shown in more detail later (Fig. 6), sodium gradient-dependent uptake is fast under these experimental conditions and reaches its maximal uptake (overshoot) prior to a 20-s incubation. This overshoot phenomenon can be taken as a first evidence for a sodium-cysteine cotransport.

Figure 2 shows that varying the osmotic pressure of the incubation medium by the addition of cellobiose reduces uptake of L-cysteine. The data are plotted as uptake against reciprocal of cellobiose concentration in the incubation medium and indicate an inverse relationship of uptake and osmolarity. At infinite cellobiose concentration there is uptake near to zero, indicating that the uptake is almost entirely a function of an osmotically sensitive intravesicular space and does not represent simple binding to the membranes. Similarly, the

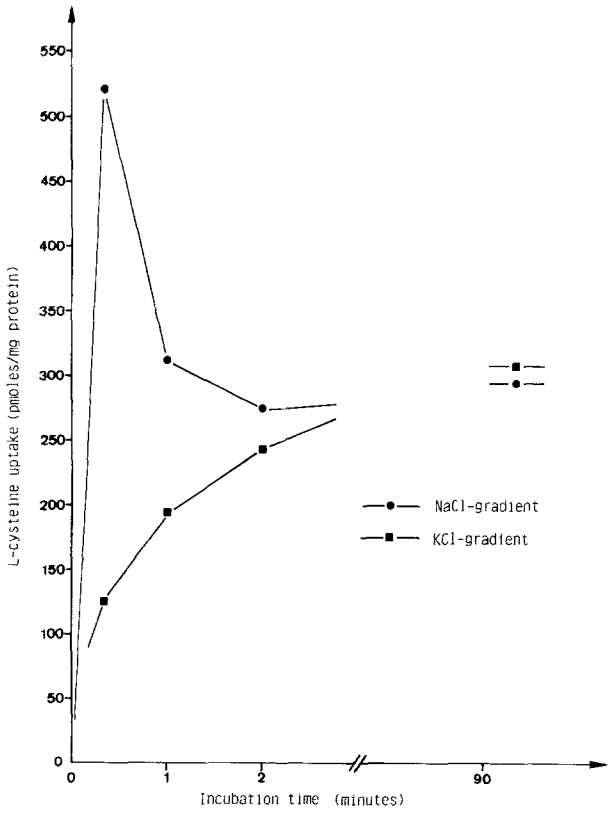


Fig. 1. Time course and sodium dependence of cysteine uptake. Membranes were loaded with 300 mmol/liter mannitol, 20 mmol/liter HEPES adjusted with Tris to pH 7.4. The incubation media contained in addition (final concentrations): 100 mmol/liter NaCl (•) or KCl (■), respectively, 0.1 mmol/liter L-^{35}S-cysteine and 1.0 mmol/liter dithiothreitol. Uptake was started by adding 20 µl of membrane suspension to 100 µl of incubation medium. At given times points 20 µl of incubation suspension were withdrawn and filtered

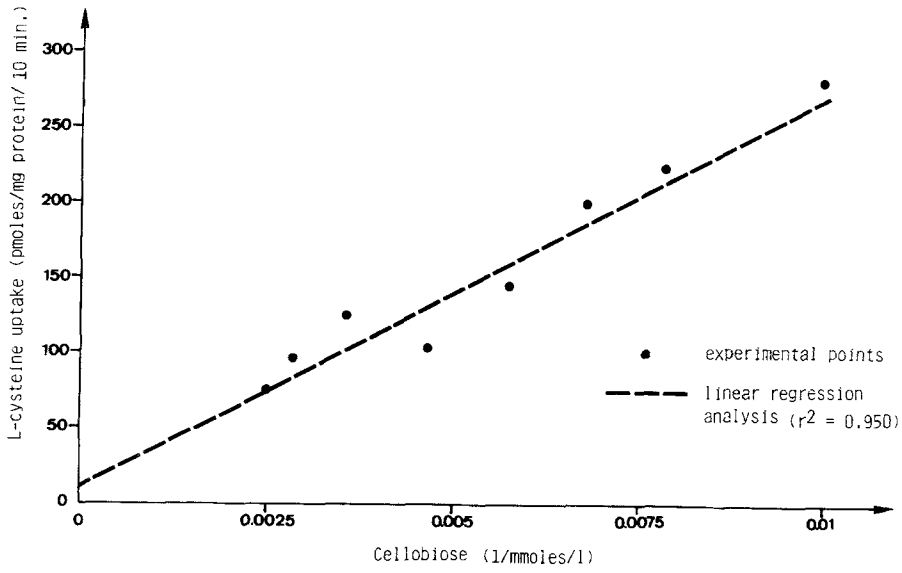


Fig. 2. Dependence of equilibrium cysteine uptake on medium osmolarity. Membranes were loaded with 100 mmol/liter mannitol, 20 mmol/liter HEPES adjusted with Tris to pH 7.4. The incubation media contained in addition (final concentrations): cellobiose (100, 127, 147, 175, 215, 280, 350 and 400 mmol/liter), 50 mmol/liter NaCl, 0.1 mmol/liter L-^{35}S-cysteine and 1.0 mmol/liter dithiothreitol. Uptake was started by adding 20 µl of membrane suspension to 80 µl of incubation medium. After 10-min incubation, 20-µl aliquots (4) were removed and filtered

Table 1. Cation specificity of stimulation of L-cysteine uptake

Salt in incubation medium (100 mmol/liter)	L-cysteine uptake (pmol/mg protein/20 sec)
NaCl	520 (325%)
LiCl	225 (140%)
KCl	155 (100%)
RbCl	165 (100%)
CsCl	155 (100%)
Choline-Cl	160 (100%)

The experimental conditions were identical to those given in the legend to Fig. 1.

observations in the rapid uptake experiments (Fig. 6) also indicate the absence of binding of L-cysteine to the external membrane surface; extrapolating the initial uptake values to a zero incubation time results in no uptake.

Ionic Requirements

In order to further investigate the cation effect on L-^{35}S-cysteine uptake, the 20-sec uptake value was determined in the presence of other alkali metals, as shown in Table 1. Only lithium seems to have a small capacity to replace sodium. From the close similarity of the uptake values in the presence of KCl, RbCl, CsCl and choline chloride, one can conclude that these monovalent ions are without an effect on L-cysteine uptake and that sodium and to a small extent also lithium, specifically stimulate L-cysteine uptake. As a potassium gradient in the presence of valinomycin is also unable to stimulate L-cysteine uptake (*data not shown*), it is likely that these effects of sodium and lithium as well as the overshoot in the presence of an inwardly directed sodium gradient (Fig. 1) are the consequences of a cotransport mechanism. A small stimulatory effect of lithium was also observed in our earlier study on L-phenylalanine uptake (Evers, Murer & Kinne, 1976).

As sodium gradient-dependent uptake of L-cysteine seems to be a very fast process (*see* Fig. 1 and Fig. 6), the effect of different anions on sodium gradient-dependent uptake of L-cysteine was measured after a 1-sec incubation period (Table 2). Uptake measured in the presence of permeant anions (thiocyanate, nitrate) is faster than the uptake measured in the presence of chloride. Uptake in the presence of relatively unpermeant anions (gluconate, isethionate, sulfate) is slow. From earlier studies with small intestinal and renal proximal tubular brush border membranes, we know that sodium-stimulated uptake of sugars and different amino acids represent electrogenic sodium-solute cotransport mechanisms (Murer & Hopfer, 1974; Kinne et al., 1975; Sigrist-Nelson, Murer & Hopfer, 1975; Evers et al., 1976; Fass, Hammerman & Sacktor, 1977; Beck & Sacktor, 1978*a*). As the anion effects on cysteine uptake seem to be related to the membrane permeability of the different anions, it can be concluded that they are also related to differences in the transmembrane anion diffusion potentials. Thus, a specific anion effect on the transport mechanism is unlikely. The finding on the anion dependence of sodium-dependent L-cysteine uptake suggests a potential-sensitive sodium L-cysteine cotransport.

Table 2. Effects of different anions on sodium-dependent L-cysteine uptake

Salt in incubation media (100 mmol/liter)	L-cysteine uptake (pmoles/mg protein)	
	1 second	90 minutes
NaCl	278	274
NaSCN	392	259
$NaNO_3$	389	278
Na-gluconate	145	264
Na-isethionate	121	297
Na_2SO_4 (50 mmol/liter + 50 mmol/liter mannitol)	122	246
KSCN	64	278
K-gluconate	60	261

Membranes were prepared in 400 mmol/liter mannitol, 20 mmol/liter HEPES adjusted with Tris to pH 7.4. In incubation media the final concentrations were 200 mmol/liter mannitol, 20 mmol/liter HEPES/Tris (pH 7.4), 0.1 mmol/liter L-cysteine, 1.0 mmol/liter DTT and salt as indicated in the Table. The uptake was performed in a semiautomatic mixing/stopping apparatus (*see* Materials and Methods). 10 µl of membrane suspension were mixed with 10 µl of incubation medium.

Influence of Membrane Potential

An effect of the membrane potential on sodium-dependent L-cysteine uptake can be tested more directly by applying potassium/valinomycin-induced membrane diffusion potentials. To provoke an inside negative diffusion potential, we preloaded the vesicles with potassium gluconate and studied L-cysteine uptake in the presence of an inwardly directed sodium gluconate gradient and in the presence or in the absence of the potassium-specific ionophore valinomycin (Fig. 3). The preloading with potassium did not significantly stimulate L-cysteine uptake as compared to a nonpreloaded control (*data not shown*). Such an effect was observed in rat and rabbit renal brush border vesicles for acidic amino acid uptake and was

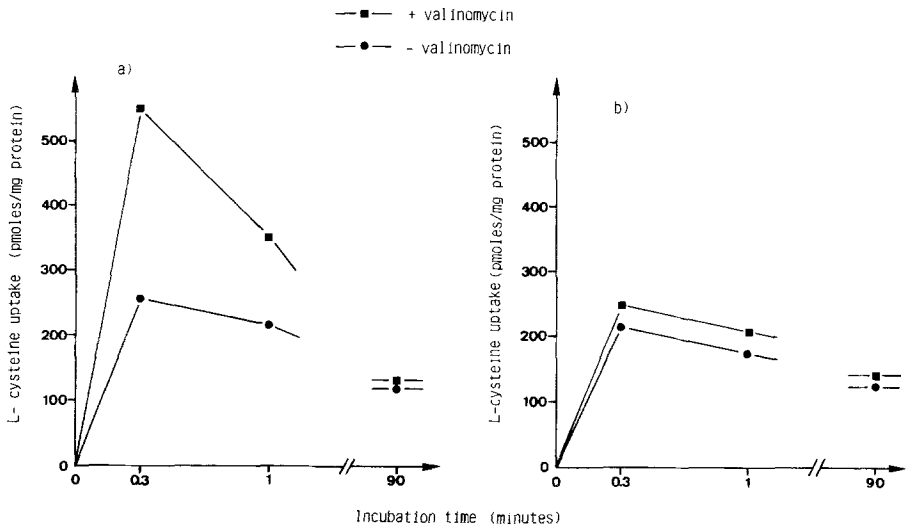


Fig. 3. Dependence of sodium-dependent cysteine uptake on potassium/valinomycin-induced diffusion potentials. Membranes were prepared in 300 mmol/liter mannitol, 20 mmol/liter HEPES adjusted with Tris to pH 7.4. One volume of the membrane suspension was mixed with 30 volumes of a buffer containing 500 mmol/liter mannitol, 20 mmol/liter HEPES-Tris and 100 mmol/liter potassium gluconate, pH 7.4. After homogenization in a glass-Teflon homogenizer and standing for 1 hr at room temperature, membranes were collected by centrifugation. Incubation media contained (final concentrations): a) 480 mmol/liter mannitol, 20 mmol/liter HEPES-Tris, pH 7.4, 10 mmol/liter potassium gluconate, 100 mmol/liter sodium gluconate, 0.1 mmol/liter L-^{35}S-cysteine and 1.0 mmol/liter dithiothreitol. *b)* 300 mmol/liter mannitol, 20 mmol/liter HEPES-Tris, pH 7.4, 100 mmol/liter sodium gluconate, 100 mmol/liter potassium gluconate, 0.1 mmol/liter L-^{35}S-cysteine and 1.0 mmol/liter dithiothreitol. The experiments were performed in the presence (■) or absence (●) of 10 μg valinomycin/100 μl incubation medium added as concentrated ethanolic solution. Final ethanol concentration in all incubation media was 1% (vol/vol). Uptake was started by adding 10 μl of membrane suspension to 110 μl incubation medium. At given time intervals, 20 μl of incubation suspension were withdrawn and filtered

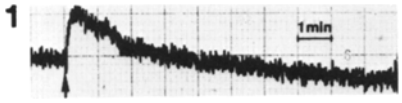
among others taken as evidence for a direct involvement of potassium flux in acidic amino acid transport (Burckhardt et al., 1980; Schneider, Hammerman & Sacktor, 1980; Schneider & Sacktor, 1980; Sacktor, Lepor & Scheider, 1981). Increasing inside negativity by adding valinomycin stimulates sodium-dependent L-cysteine uptake markedly (Fig. 3*a*). This stimulation is not observed in the absence of sodium (*not shown*). Furthermore, an abolishment of the potassium gradients by adding the same potassium concentration outside as well as inside, prevents this valinomycin effect (Fig. 3*b*). Similar effects were reported for small intestinal and renal proximal tubular transport of glucose and neutral amino acids (e.g. Murer & Hopfer, 1974; Kinne et al., 1975; Sigrist-Nelson et al., 1975; Evers et al., 1976; Fass et al., 1977; Beck & Sacktor, 1978*a*).

We also tested the effect of sodium-dependent L-cysteine uptake on the transmembrane diffusion potential (Fig. 4). As documented in earlier studies, the positively charged fluorescent cyanine dye 3,3′-diethylthiadicarbocyanine iodide can be successfully used in renal brush border vesicles to monitor transport-dependent membrane potential changes (Beck & Sacktor, 1978*b*; Burckhardt & Murer, 1981; Wright et al., 1981). Addition of D-glucose to sodium sulfate pre-equilibrated membrane vesicles provokes a transient increase in fluorescence (Fig. 4*a*,1). As can be seen from the signal obtained by potassium injection in the presence of valinomycin, this transient increase in fluorescence corresponds to an increased inside-positive transmembrane diffusion potential (Fig. 4*a*,2). The presence of dithiothreitol does not alter the D-glucose-induced fluorescence change (Fig. 4*a*,3) indicating an absence of unspecific effects of this drug. In the absence of sodium, in potassium pre-equilibrated membrane vesicles, D-glucose addition does not provoke a fluorescence signal (Fig. 4*a*,4). The addition of L-cysteine to sodium pre-equilibrated vesicles transiently increases the fluorescence, suggesting that L-cysteine influx under these conditions is also accompanied by an influx of positive charges (Fig. 4*b*,5). Under our experimental conditions, the L-cysteine-dependent fluorescence change is higher in magnitude and faster than the D-glucose signal. An increase in the conductance for cations by the addition of gramicidin D prevents the L-cysteine-dependent fluorescence change (Fig. 4*b*,6). This finding documents that the signal observed in the absence of the ionophore (Fig. 4*b*,5) is not due to unspecific effects. Different from the D-glucose (Fig. 4*a*,4), L-cysteine addition

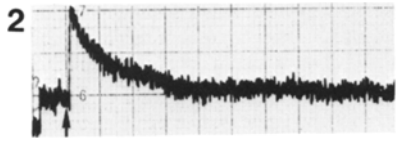
a) Controls (D-Glucose-signals; Potassium/valinomycin signal)

sodium equilibrated:

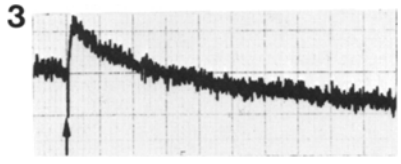
1) D-Glucose addition: 10.8 mmoles/l



2) potassium addition in the presence of valinomycin: 7.5 mmoles/l

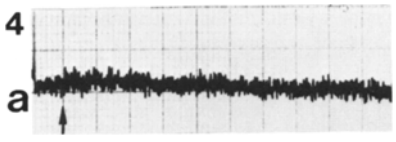


3) D-glucose addition in the presence of DTT: 10.8 mmoles/l



potassium equilibrated:

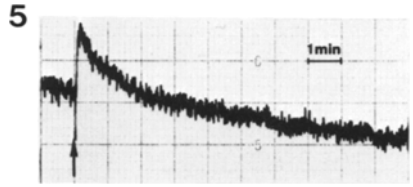
4) D-glucose addition: 10.8 mmoles/l



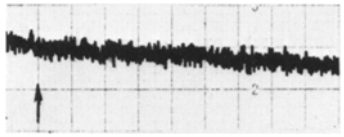
b) L-cysteine (in the presence of DTT)

sodium equilibrated:

5) L-cysteine addition: 47.6 mmoles/l

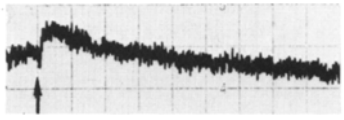


6) L-cysteine addition in the presence of gramicidin D: 47.6 mmoles/l

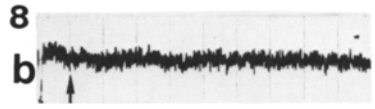


potassium equilibrated:

7) L-cysteine addition: 47.6 mmoles/l



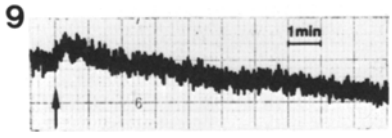
8) L-cysteine addition in the presence of gramicidin D: 47.6 mmoles/l



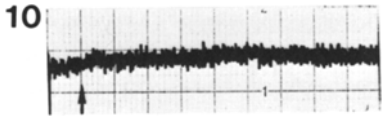
c) D-cysteine (in presence of DTT)

sodium equilibrated:

9) D-cysteine addition: 47.6 mmoles/l

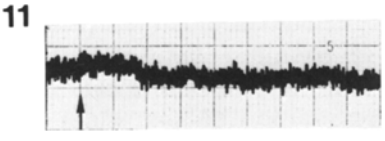


10) D-cysteine addition in the presence of gramicidin D: 47.6 mmoles/l



potassium equilibrated:

11) D-cysteine addition: 47.6 mmoles/l



12) D-cysteine addition in the presence of gramicidin D: 47.6 mmoles/l

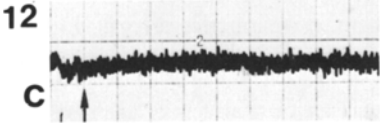


Fig. 4. Effect of L/D-cysteine addition on the fluorescence of 3,3′ diethylthiadicarbocyanine iodide. Membranes were prepared in either 74 mmol/liter Na_2SO_4, 1 mmol/liter K_2SO_4, 20 mmol/liter HEPES-Tris, pH 7.4 (buffer A) or in 75 mmol/liter K_2SO_4, 20 mmol/liter HEPES adjusted with Tris to pH 7.4 (buffer B): The solutes to be added during the experiment to the cuvette were solubilized either in buffer A or in buffer B. For all experiments 250 µg of membrane protein was used per cuvette. Dye concentration was 3 µmol/liter. *a) Controls*: 1) 2 ml buffer A, 20 µl of membranes in buffer A, 50 µl D-glucose (final concentration: 10.8 mmol/liter); 2) 2 ml buffer A, 20 µl of membranes in buffer A, 10 µl valinomycin (final concentration: 5 µg/ml), 200 µl K_2SO_4 (final concentration: 7.6 mmol/liter); 3) Identical to 1): 20 µl dithiothreitol was added (final concentration: 5 mmol/liter); 4) identical to 1): instead of buffer A, buffer B was used. *b) L-cysteine*. 5) 2 ml buffer A, 20 µl of membranes in buffer A, 20 µl dithiothreitol (final concentration: 5 mmol/liter), 100 µl L-cysteine (final concentration: 47.6 mmol/liter); 6) Identical to 5): addition of 10 µl gramicidin D (final concentration: 5 µg/ml); 7) Identical to 5): instead of buffer A, buffer B was used; 8) L-cysteine addition in the presence of gramicidin D: 47.6 mmol/liter. Identical to 6): instead of buffer A, buffer B was used; *c) D-cysteine*: 9) 2 ml buffer A, 20 µl of membranes in buffer A, 20 µl dithiothreitol (final concentration: 5 mmol/liter), 100 µl D-cysteine (final concentration: 47.6 mmol/liter); 10) Identical to 9): addition of 10 µl gramicidin D (final concentration: 5 µg/ml); 11) Identical to 9): instead of buffer A, buffer B was used; 12) Identical to 10): instead of buffer A, buffer B was used. ↑ Indicates time point of substrate injection into the cuvette

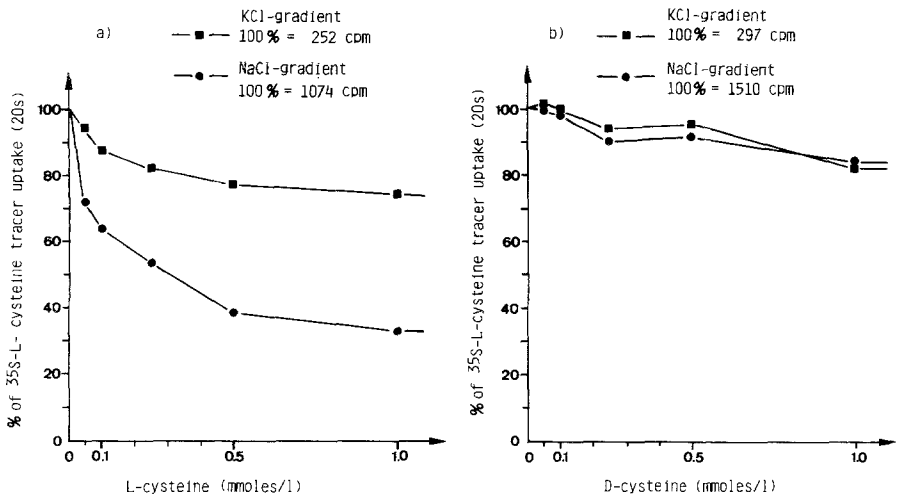


Fig. 5. Stereospecificity of cysteine uptake; tracer replacement with unlabeled L-cysteine (*a*) or D-cysteine (*b*). Membranes were prepared in 300 mmol/liter mannitol, 20 mmol/liter HEPES adjusted with Tris to pH 7.4. The incubation media contained in addition (final concentrations) 100 mmol/liter NaCl (●) or KCl (■), tracer L-^{35}S-cysteine (tracer concentration: 0.02219 μmol/liter) and 5 mmol/liter dithiothreitol. The final concentrations of L-cysteine (*a*) or D-cysteine (*b*) are given in the Figure. Uptake was started by adding 20 μl of membrane suspension to 40 μl of incubation medium. After 20 sec of incubation 20 μl of membrane suspension were withdrawn and filtered

under potassium-equilibrated conditions induces also a fluorescence signal (Fig. 4*b*,7). This signal is considerably smaller than in the sodium medium and is again completely prevented by the addition of gramicidin D (Fig. 4*b*,8). A conclusive interpretation of this finding is difficult. It could be possible that the injected concentrated L-cysteine solution contains small contaminations with sodium, which would be sufficient to stimulate a L-cysteine-dependent inside-positive diffusion potential. An unlikely explanation would be a partial replacement of sodium by potassium as a cosubstrate in the cotransport mechanism. Addition of D-cysteine to sodium-pre-equilibrated membranes provokes a fluorescence change (Fig. 4*c*,9), which is much smaller than the corresponding L-cysteine signal (Fig. 4*b*,5). The D-cysteine signal is also prevented by the addition of gramicidin D (Fig. 4*c*,10). Addition of D-cysteine to potassium-equilibrated vesicles is unable to produce a significant fluorescence change (Fig. 4*c*,11 and Fig. 4*c*,12). In summary, comparing the L-cysteine effects observed with sodium-equilibrated vesicles (Fig. 4*b*,5 and 4*b*,6) with those obtained with potassium-equilibrated vesicles (Figs. 4*b*,7 and 4*b*,8), it is evident that L-cysteine is preferentially transported in a sodium-containing medium in an electrogenic mode. For the fluorescence signal for L-cysteine in the potassium medium, we are unable to present a satisfactory explanation (*see above*). For D-cysteine, the situation is analogous to the situation for L-cysteine, although much smaller signals are observed. Thus, a comparison of the results obtained with L-cysteine and D-cysteine indicates a preferential transport of L-cysteine, i.e. the system operates stereospecifically. D-cysteine might also be transported, but with a much smaller rate than L-cysteine (*see also below*).

Substrate Binding

The question related to sodium dependence and stereospecificity of the transport mechanism was further investigated. As labeled D-cysteine is not easily available, we decided to perform a tracer competition experiment with both stereoisomers by using only labeled L-^{35}S-tracer substrate (Fig. 5). Tracer uptake in the presence of an inwardly directed sodium gradient is strongly reduced by increasing unlabeled L-cysteine at concentrations up to 1.0 mmol/liter (Fig. 5*a*) and also slightly affected by using the D-isomer in the same concentration range (Fig. 5*b*). These findings suggest that D-cysteine, although to a much smaller extent, interacts with the transport pathway. Increasing D-cysteine concentration up to 5.0 mmol/liter reduces the uptake of tracer L-cysteine to 68% in the sodium medium (*data not shown*). For the L-isomer, some tracer replacement is also observed under potassium gradient condition (Fig. 5*a*). The D-cysteine effect in the presence of a potassium gradient is indistinguishable from the L-cysteine effect in the concentration range up to 1.0 mmol/liter (Fig. 5*ab*). At a D-cysteine concentration of 5.0 mmol/liter, L-cysteine tracer uptake was 77% of the control value in the potassium medium (*data*

Table 3. Inhibition of sodium-dependent L-cysteine (0.1 mmol/liter) uptake by different amino acids

Amino acid in incubation medium (2.5 mmol/liter)	L-cysteine uptake (pmol/mg protein)	
	10 seconds	90 minutes
none	447 (100%)	279
D-glucose	402 (90%)	284
L-alanine	282 (63%)	272
L-phenylalanine	294 (65%)	274
L-glutamic acid	414 (93%)	295
L-arginine	384 (86%)	297
L-ornithine	440 (98%)	288
L-lysine	441 (98%)	277

Membranes were prepared in 200 mmol/liter mannitol, 20 mmol/liter HEPES adjusted with Tris to 7.4 and 100 mmol/liter KCl. The incubation medium contained in addition (final concentrations): 100 mmol/liter NaCl, 0.1 mmol/liter L-^{35}S-cysteine, 1.0 mmol/liter dithiothreitol and 2.5 mmol/liter of glucose or different amino acids as indicated in the Table. Uptake was started by adding 20 µl of membrane suspension to 80 µl of incubation medium. Valinomycin was added as an ethanolic solution. (Final concentration: 1% ethanol and 10 µg valinomycin/100 µl incubation medium.)

not shown). This observation could indicate a weak sodium dependence also in the D-cysteine experiments.

On the basis of these tracer replacement experiments we can assume that cysteine is transported across the brush border membrane by a sodium-dependent stereospecific transport mechanism. For L-cysteine, tracer replacement is more pronounced in a sodium medium than in a potassium medium. The sodium-independent tracer replacement effect could be evidence for a sodium-independent pathway for L-cysteine. Although some tracer replacement was observed for D-cysteine, no firm conclusions about D-cysteine transport can be made from this experiment. In particular, the extremely weak sodium dependence of this tracer replacement is not understood. A speculative interpretation could relate this observation to the existence of a low affinity, probably sodium-independent; transport pathway accepting neutral amino acids with little or no stereospecificity. In this respect, firm conclusions can certainly not be reached from experiments with vesicles, as small cross-contaminations of brush border membrane preparations by other membrane vesicles (e.g. basal-lateral) could also contribute to such findings.

The sodium-dependent transport of L-cysteine is inhibited by neutral L-amino acids, but not by L-acidic and L-basic amino acids (Table 3). This finding suggests that sodium-dependent cysteine uptake shares transport systems involved in the

Table 4. Transstimulation of L-^{35}S-tracer uptake by L-cysteine, L-alanine and L-phenylalanine

Amino acid concentration (in mmol/liter)		L-^{35}S-cysteine uptake (in cpm)			
Incubation medium	Pre-equilibration	Time of incubation			
		2 s	5 s	15 s	90 min
0.1 L-cysteine	–	744	933	1105	3150
0.1 L-cysteine	1.0 L-cysteine	1140	1262	1345	3068
0.1 L-cysteine + 0.1 L-alanine	–	589	767	977	2958
0.1 L-cysteine + 0.1 L-alanine	1.0 L-alanine	832	1090	1269	3046
0.1 L-cysteine + 0.1 L-phenylalanine	–	662	772	858	3074
0.1 L-cysteine + 0.1 L-phenylalanine	1.0 L-phenylalanine	862	1040	1314	2991

Membranes have been prepared in 400 mmol/liter mannitol, 20 mmol/liter HEPES adjusted with Tris to pH 7.4, 20 mmol/liter KCl and 100 mmol/liter NaCl. Membranes have been pre-equilibrated for 1 h at room temperature in the presence of 0.1 mg/ml valinomycin and in the presence or absence of either 1.0 mmol/liter of L-cysteine, L-alanine or L-phenylalanine, respectively. Uptake of L-^{35}S-tracer was studied with the rapid uptake apparatus by mixing 5 µl of pre-equilibrated membrane suspension with 45 µl of incubation medium. The incubation media contained 400 mmol/liter mannitol, 20 mmol/liter HEPES Tris pH 7.4, 20 mmol/liter KCl, 100 mmol/liter NaCl, 0.1 mg/ml valinomycin, 1.0 mmol/liter dithiothreitol and the amino acids as indicated in the Table (final concentrations after mixing of membranes with incubation medium).

transport of neutral amino acids. In this type of experiment, special precautions have to be taken in order to prevent indirect interactions related to changes in driving forces for different sodium-dependent transport mechanisms (Murer, Sigrist-Nelson & Hopfer, 1975; Thierry, Poujeol & Ripoche, 1981). In our experiments, membrane potential-related inhibitions have been minimized by short-circuiting the membrane potential with potassium and valinomycin. However, from the data obtained on L-cysteine uptake in the presence of D-glucose, it can be seen that indirect inhibition cannot be completely prevented by this maneuver. Increased tracer L-cysteine uptake is observed after preloading the vesicles with L-phenylalanine or L-alanine (Table 4). As this observation is made under conditions of a membrane-potential short circuit, it can only be explained by an identical transport pathway for these different neutral amino acids.

Finally, we made attempts to measure the kinetic parameters of sodium-dependent L-cysteine uptake under initial linear flux conditions. Under

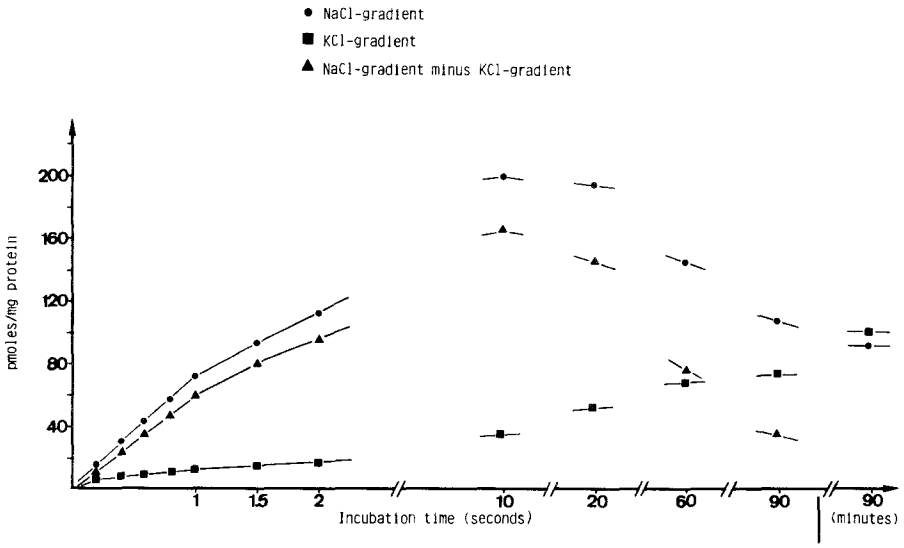


Fig. 6. Initial, linear uptake of L-cysteine. Membranes were prepared in 400 mmol/liter mannitol, 20 mmol/liter HEPES adjusted with Tris to pH 7.4. The incubation medium contained 200 mmol/liter KCl (■) or NaCl (●), 0.2 mmol/liter L-^{35}S-cysteine, 10 mmol/liter dithiothreitol and 20 mmol/liter HEPES-Tris (pH 7.4). Except for HEPES-Tris final concentrations were half (mixing 1:1 of membranes and incubation medium). The uptake was performed in a semiautomatic mixing/stopping apparatus (*see* Materials and Methods). Ten µl of membrane suspension were mixed with 10 µl of incubation medium. (▲) NaCl gradient-KCl gradient

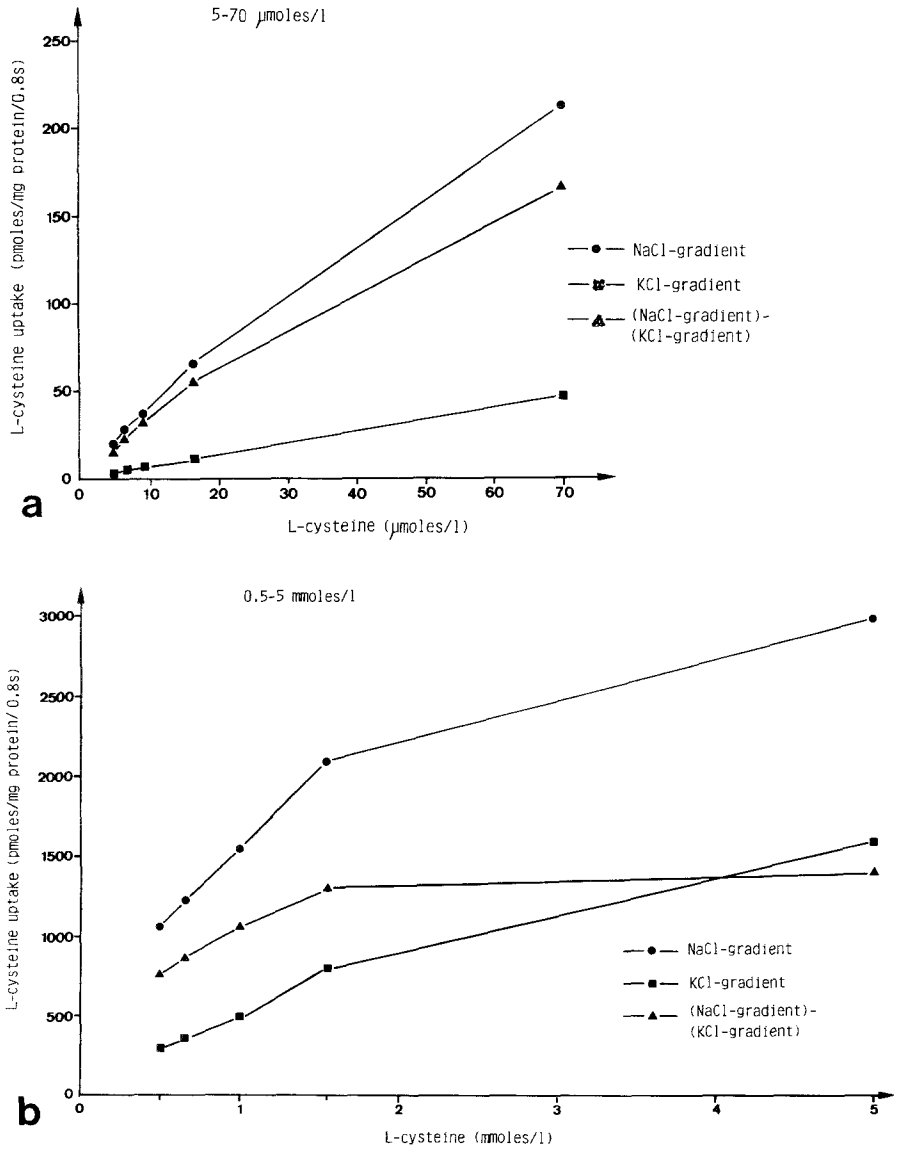


Fig. 7. Saturation of initial L-cysteine uptake. The experimental conditions are identical to those given in the legend to Fig. 6. As indicated in the Figure, L-cysteine concentration was varied between 5 and 70 µmol/liter (*a*) and 0.5 to 5.0 mmol/liter (*b*). Experimental time is 0.8 s. (●) NaCl gradient condition; (■) KCl gradient condition; (▲) NaCl gradient-KCl gradient

Table 5. Kinetic parameter of L-cysteine uptake as obtained by iterative stripping computer analysis of S/v (substrate concentration/uptake at 0.8 s per mg of protein) values plotted against substrate concentration (Hanes-Woolf plot). The experimental conditions are identical to those given in the legend to Fig. 6 and 7

Experiment	Concentration range tested	Apparent affinity constant (K_m) (mol/liter)	Apparent maximal velocity (v_{max}) (mol/mg protein/0.8 sec)	Reliability of data (r^2)
1	5–70 μmol/liter (NaCl gradient)	28.4×10^{-6}	58.6×10^{-12}	0.9921
	0.5–5 mmol/liter (NaCl gradient-KCl gradient)	894×10^{-6}	2110×10^{-12}	0.9991
2	5–125 μmol/liter (NaCl gradient)	89.2×10^{-6}	142×10^{-12}	0.9896
	5–125 μmol/liter (NaCl gradient-KCl gradient)	77.2×10^{-6}	107×10^{-12}	0.9883
	0.5–5 mmol/liter (NaCl gradient-KCl gradient)	1330×10^{-6}	1060×10^{-12}	0.9988
3	5–125 μmol/liter (NaCl gradient)	66.1×10^{-6}	76×10^{-12}	0.9975
	0.5–5 mmol/liter (NaCl gradient)	1500×10^{-6}	1580×10^{-12}	0.9998

sodium-gradient and potassium-gradient conditions, linear uptake was only observed within the first second (Fig. 6). Control experiments indicated that also at the highest substrate concentration used (5 mmol/liter), linear uptake was observed within the first second of incubation (*data not shown*).

For our experiments we therefore decided to use an 0.8 s incubation time. As preliminary experiments suggested saturation of sodium gradient-dependent L-cysteine uptake at μmolar as well as at mmolar concentrations, we tested these two concentration ranges and analyzed the data by iterative stripping computer analysis (Spears et al., 1971). Figures 7*a* and 7*b* show the saturation behavior in the two concentration ranges tested. Partial saturation also occurs at the lower concentration. Complete saturation is obtained in the mmolar concentration range. Furthermore, these data indicate that for a kinetic analysis the values obtained under sodium gradient conditions should be corrected by the potassium values, at least in the mmolar concentration range. In the low concentration range a correction would also be ideal, but is experimentally difficult to perform. At this low substrate concentration the low potassium values can be determined very inaccurately, due to relative high and inconsistent background corrections. This problem does not occur at the high concentration range tested. This experimental difficulty is the explanation for presenting in Table 5 for the high concentration range the data for the corrected values, whereas for the low concentration range there is presented mostly an analysis of the uncorrected values. As can be seen from the data, we have evidence for two transport mechanisms. The low affinity system has an apparent affinity constant around 1 mM whereas a system with higher affinity shows an apparent affinity constant below 100 μM. The capacity of the low affinity system is at least 10 times that of the high affinity system.

The saturation phenomenon (Figs. 7*a* and 7*b*, Table 5) is partially due to indirect effects related to changes in membrane potential (altering driving forces) as a consequence of increased sodium-coupled flux by increasing substrate concentrations. Control experiments performed in the presence of equal potassium concentrations on both membrane sites in the presence and absence of valinomycin documented such "indirect" saturation effects. Measured at a substrate concentration of 0.1 mmol/liter, a "membrane potential short circuit" by the addition of valinomycin caused a 10 to 15% stimulation of sodium gradient-dependent L-cysteine uptake; at a concentration of 5.0 mmol/liter the addition of valinomycin provoked a 25 to 30% stimulation (*data not shown*). These indirect (membrane potential-mediated) saturation effects certainly "falsify" the values presented (Table 5). However, they are too small to explain the observed saturation at the two concentration ranges tested. Nevertheless, these controls indicate that kinetic parameters obtained with vesicles are strongly dependent on the experimental conditions used. Thus, the values given in Table 5 can only be taken as evidence for two transport pathways; the values presented in Table 5 for the

kinetic parameters are certainly valid only for the experimental conditions used and only provide semiquantitative information on the different affinities and capacities of the two postulated transport pathways for L-cysteine.

Discussion

The data presented above document a stereospecific, sodium-dependent, potential-sensitive uptake of cysteine into rat renal proximal tubular brush border membrane vesicles. This is in complete agreement with electrophysiological data obtained by Samarzija and Frömter (1982). No final answers can be given for the multiplicity of the transport mechanism as well as for the selectivity of these transport mechanisms with regard to other amino acids.

On the basis of our kinetic analysis we postulate the existence of two transport mechanisms for L-cysteine. However, there is no experimental evidence available that these two sodium-dependent transport mechanisms are located within the same vesicular membrane. First, they could reflect different brush border vesicle populations, i.e. vesicles from superficial nephrons and vesicles from juxtamedullary nephrons. It is known that proximal tubular transport functions can show at least quantitative differences in differently located nephrons (e.g., Haramati & Knox, 1981; Turner & Dousa, 1982; Turner & Moran, 1982). Also different transport properties in the *pars recta* as compared to early and late proximal tubule might be an explanation. This uncontrolled vesicle heterogeneity is generally not taken into consideration and makes it very difficult to interpret the kinetic data with vesicles. Also small contaminations with basal-lateral membrane might represent a problem. It is known that also in basal-lateral membranes sodium-dependent pathways for neutral amino acids exist (Mircheff, van Os & Wright, 1980).

The absolute values for the kinetic parameters are strongly dependent on the experimental conditions used, e.g. sodium-equilibrated or sodium gradient conditions (Geck & Heinz, 1976; Hopfer, 1977; Tannenbaum et al., 1977; Toggenburger et al., 1978; Turner & Silverman, 1978, 1980). We believe that a determination under sodium gradient conditions reflects more the physiological situation than a determination under sodium-equilibrated conditions. However, a closer characterization of the effective parameters of the transporter molecule could be obtained in zero net flux tracer exchange conditions in the absence of electrochemical sodium gradients (Hopfer & Groseclose, 1980). For a determination of the "apparent" transport parameters under sodium gradient conditions, initial linear uptake values are mandatory for an analysis according to simple Michaelis-Menten kinetics. For most of the previously published kinetic parameters of amino acid transport in brush border membrane vesicles, initial linear influx conditions have not been established (e.g., Evers et al., 1976; Fass et al., 1977; Segal et al., 1977; Schneider et al., 1980; McNamara et al., 1981; Weiss et al., 1981). With the introduction of a semiautomatic apparatus for such uptake studies, it has now become possible to analyze the "apparent" kinetic parameters under the appropriate conditions (Kessler et al., 1978; Stevens et al., 1982).

The fluorescence experiments are best performed under sodium-equilibrated conditions; in the presence of transmembrane salt gradients the fluorescence base-line is unstable, due to continuous changes in the transmembrane salt gradient-dependent diffusion potentials. From tracer studies on intestinal vesicles it is known that the "apparent" affinity constant of sodium-dependent D-glucose uptake measured under sodium-equilibrated conditions is two orders of magnitude higher than measured under sodium gradient conditions (Hopfer, 1977; Kessler et al., 1978; Toggenburger et al., 1978). Similarly, also in renal vesicles the size of the kinetic parameters is strongly dependent on the experimental conditions used. Thus, it is certainly not surprising that we had to use high cysteine concentrations for the experiments with the fluorescent dye in order to obtain a reasonable signal-to-noise ratio.

A recent study on amino acid transport by rabbit kidney brush border vesicles provided evidence for six different transport mechanisms involved in neutral amino acid uptake (Mircheff et al., 1982). In that study, the transport of L-cysteine was not tested. The experiments described in this paper show inhibition of L-cysteine by L-alanine and L-phenylalanine but not by L-lysine. Taking these data together with those presented by Mircheff et al. (1982), four different transport mechanisms would be candidates for L-cysteine transport (Mircheff et al., 1982; Table 8; this publication, Table 3). Nevertheless, we have evidence for only two transport mechanisms by kinetic arguments. Thus, there is a multiplicity of transport mechanisms for neutral amino acids. At present it is not possible to decide about the number of different transport mechanisms for neutral amino acids accepting also L-cysteine as well as about their tubular localization. For experimental reasons, we do not think that only studies with vesicles

can give the answer, unless it would be possible to define more closely the vesicle population with respect to its tubular origin. Electrophysiological experiments might help to complete our information on L-cysteine transport. As such studies, however, are not well-suited for the detection of high affinity/low capacity transport mechanisms, it is not too surprising that a multiplicity for transport mechanism for L-cysteine was not described (Samarzija & Frömter, 1982). Electrophysiological studies indicated that L-cysteine shares the transport pathway for neutral amino acids. In apparent disagreement with our data on the vesicles are recent data obtained in *in vivo* experiment. L-methionine but not L-phenylalanine inhibited L-cysteine transport (Völkl & Silbernagl, 1982).

Several questions with respect to transport of cysteine remain open. As outlined above, the question related to the driving forces for cysteine transport seems to be answered. A response to the questions related to a multiplicity of transport mechanisms as well as to substrate specificity requires further experimentation. Moreover, in order to understand the different forms of cystinuria, we have to also analyze transport of cystine and must distinguish it clearly from that of cysteine. Our preliminary experiments indicate that transport of cystine is separate from transport of cysteine and occurs via sodium-dependent and sodium-independent pathways shared also by the basic amino acids (Biber et al., 1981*b*).

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