Biochimica et Biophysica Acta 859 (1986) 79–87
Elsevier

BBA 73141

L-Cystine transport by papain-treated rat renal brush-border membrane vesicles

Betty Y.L Hsu, Susan M Corcoran and Stanton Segal *

Division of Biochemical Development and Molecular Diseases The Children's Hospital of Philadelphia and The Departments of Pediatrics and Medicine University of Pennsylvania School of Medicine, Philadelphia, PA 19104 (U S A)

(Received February 18th, 1986)

Key words Brush-border membrane Amino acid transport, L-Cystine transport, Membrane potential, (Rat kidney)

In papain-treated rat renal brush-border membrane vesicles, cystine uptake was enhanced under sodium gradient conditions. This effect was not observed when sodium was equilibrated across the vesicle membrane or when sodium was completely absent from the incubation medium. The increased rate of cystine uptake occurred within the first two minutes of incubation and coincided with the period of increased flux of sodium known to occur after papain treatment. Under sodium gradient conditions, the V_{max} of cystine uptake by treated vesicles was 65% greater while the K_m was 25% lower than the value observed in untreated membranes. The increased cystine uptake after papain treatment occurred when medium cystine was in the electroneutral form. In the absence of a sodium gradient, cystine uptake by control membranes was insensitive to changes in membrane potential and this was unaltered after papain treatment. Exposure of the membranes to papain also resulted in a profound decrease in cystine binding which occurs in native membranes incubated with cystine. The fact that cystine uptake is unchanged under sodium equilibration and even enhanced under sodium gradient conditions suggests that the component of cystine binding is not essential for cystine transport and may represent non-specific binding to membrane proteins.

Introduction

Proteases have been used widely to study the relationship of the asymmetric properties of brush-border membrane vesicles to specific transport properties [1,2] Our own interest has focused on whether agents that remove enzymes and proteins from rat renal brush-border membranes alter the ability of membrane vesicles to transport amino acids and, if so, what is the underlying mechanism Studies with trypsin and papain treatment indicate that removal of small amounts of protein may have profound effects on sodium-dependent transport [3,4] A detailed study of proline transport changes associated with papain treatment revealed that the 'overshoot' phenomena observed with a sodium gradient was markedly blunted with no impairment of proline uptake under sodium equilibrated conditions The renal brush-border uptake of a number of amino acids and sugars under sodium gradient conditions was also decreased, consistent with a primary perturbation of sodium ion movement Indeed, this was shown to be the case Papain treatment caused an enhancement of sodium fluxes so that equilibration of the sodium gradient occurred five times more rapidly We were led to conclude that papain affects sodium gradient stimulated transport secondarily because of the accelerated dissipation of sodium gradients by treated vesicles

Cystine uptake by rat renal brush-border mem-

* To whom correspondence should be addressed at The Children's Hospital of Philadelphia, Division of Biochemical Development and Molecular Diseases, 34th and Civic Center Boulevard, Philadelphia, PA 19104, U S A

brane vesicles occurs by a process distinct from most other amino acids Although it's entry is stimulated by sodium, there is no 'overshoot' observed In addition, cystine uptake is characterized by 60% of binding to the membrane under sodium equilibrated condition, a phenomenon not observed with other amino acids [7] Because of the unique features of cystine transport by brush-border membrane vesicles and the opportunity to enhance our understanding of the complex process of cystine entry, we have examined the effect of papain treatment of rat renal membranes on cystine transport Our findings that cystine uptake rate is stimulated in papain-treated vesicles and that cystine binding to the brush-border is markedly decreased form the basis of this report

Experimental

Preparation of rat renal brush-border membranes and papain-treatment of the isolated renal brush-border vesicle membranes Brush-border membranes were isolated from the kidney cortex of adult male Sprague-Dawley rats weighing 250–300 g by using the $MgCl_2$ precipitating method of Booth and Kenny [5] Papain treatment of the membrane vesicles was carried out as described previously [4] The final papain-treated membrane preparation was suspended in buffer as specified in the legend of figures and tables, and equilibrated for at least 1 h at 22°C before undertaking any experiment Control membrane vesicle preparations were carried out by replacing aliquots of papain solution with buffer used in the incubation All experiments consisted of both control and papain treated preparations

Enzyme assays and protein concentration determination Papain treatment was monitored by assessment of the removal of membrane enzymes The five marker enzymes measured were alkaline phosphatase, L-leucine aminopeptidase, gamma-glutamyltranspeptidase, gamma-glutamylhydrolase and alpha-D-glucosidase according to the procedures reported previously [4] Membrane protein concentration was determined by the method of Bradford [6] using the Bio-Rad protein assay kit from Bio-Rad, Rockville Center, NY

Uptake of L-cystine The standard uptake incubation consisted of 0 05 ml of brush-border vesicles in buffer A (2 mM Tris-Hepes buffer (pH 7 4) containing 100 mM mannitol) or otherwise specified, which was added to a disposable test tube containing 50 μmoles of NaCl, 5 μl of unlabelled L-cystine of the desired concentration in 0 5 M HCl, 0 2 to 0 8 μCi of L-[^{35}S]cystine, and/or 0 6 μCi of L-[^{3}H]glucose, 0 50 ml of buffer B (20 mM Tris, 20 mM Hepes and 60 mM mannitol), and water The final incubation was 88 5 mM with respect to NaCl with 0 075–0 150 mg of membrane protein in a final volume of 0 565 ml

The pH was checked after mixing of 5 μl of L-cystine in 0 5 M HCl, with buffer The ratio of HCl to buffer is 1 113 under base line conditions, that is not sufficient to change the pH of the buffer system For various L-cystine concentration studies, the L-cystine was made from one single 10 mM L-cystine stock which was dissolved in 0 5 M HCl Therefore 5 μl of 0 5 M HCl was contained in each sample volume of 0 565 ml while the L-cystine concentration were varied

Uptake at 22°C was measured by rapid filtration on Sartorius filters (SM 113, 0 45 μm) as described by McNamara et al [7] Total uptake was measured as membrane-associated radioactivity present on the filter with appropriate background subtracted Where uptake in the absence of Na^+-gradient was to be studied, 0 05 ml of membrane vesicles was preincubated with 88 5 mM NaCl and 0 50 ml of buffer B in a final volume of 0 550 ml for at least 60 min before 15 μl of L-[^{35}S]cystine of desired concentration and/or L-[^{3}H]glucose was added

The extent of L-[^{35}S]cystine bound to membrane vesicles was assessed according to the procedure of McNamara et al [7] Trichloroacetic acid was added to a final concentration of 10% (w/v) to incubated samples at the designated times The samples were placed in ice for at least 15 min before being filtered through glass fiber filters (No 934AH, Reeve Angel, Clifton, NJ, U S A) in a Sartorius filter apparatus The filtered precipitates were washed with 10 ml of 5% trichloroacetic acid and then 10 ml of ethanol Filters were air dried, placed in 4 ml of standard scintillation fluid in glass mini-vials and measured for radioactivity Net uptake, the uptake of 'free' or unbound cystine, was obtained by subtracting the amount

of L-[^{35}S]cystine precipitated with trichloroacetic acid from the total uptake

The effect of osmotic perturbation on vesicle uptake of L-cystine and L-glucose was examined by adding sucrose to buffer B in the standard incubation mixture in order to vary the osmolality of the medium The incubation medium contained buffer B and was 0–280 mosM with respect to sucrose Brush-border vesicles were allowed to equilibrate for 30 min in the incubation media before uptake of labelled substrate was measured

Materials The L-[^{35}S]cystine used in these experiments was obtained from Amersham (SJ-126 Batches 6/82, 11/82, 7/83, 2/84, 4/84) and New England Nuclear Corp (NEG-020 Lot No 1622-021) All of the L-[^{35}S]cystine used in these studies was checked for purity by thin-layer chromatography according to the procedure described by States and Segal [8] and/or high-voltage electrophoresis using a Camag apparatus as reported by States et al [9]

L-[^{3}H]Glucose and D-[^{14}C]glucose were purchased from New England Corp Unlabelled amino acids, sugars and valinomycin were obtained from Sigma Hepes was from Calbiochem

Data analysis In studies of concentration dependence of uptake of the substrates by renal brush-border membrane vesicles, net uptake data were plotted by the Lineweaver-Burk method and the data fitted by the least-squares method The apparent K_m and V_{max} values were determined for each experiment

Results

Effect of papain on cystine uptake in response to osmotic perturbation of membrane vesicles

McNamara et al [7] demonstrated that cystine uptake was considerable when the brush-border vesicle space was theoretically obliterated at infinitely high osmolality and concluded that adsorption of cystine to the membrane contributed greatly to the total uptake of cystine This was corroborated by the precipitation of significant amounts of cystine when membranes were treated with trichloroacetic acid [7] The uptake of cystine after osmotic perturbation was, therefore, examined to determine the extent of cystine entry into the intravesicular space and the apparent

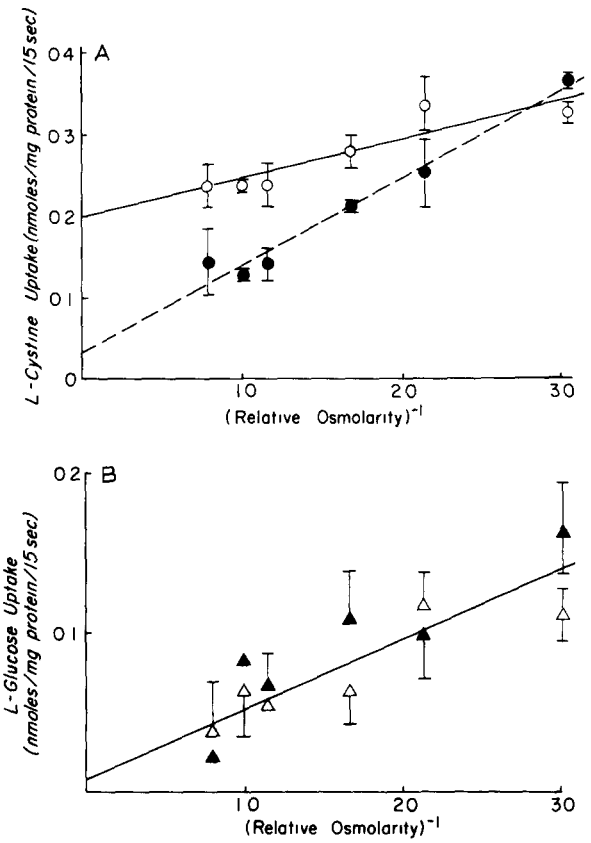

Fig 1 Effect of medium osmolality upon the uptake of L-cystine and L-glucose by control and papain-treated vesicles Control (○, △) and papain-treated (●, ▲) vesicles were obtained by incubation at 37°C for 10 min in the absence or presence of 0 015 mg activated papain per ml of membrane preparation At the end of incubation, the membrane fractions were centrifuged and washed as described in the text Membrane fractions were suspended in 100 mM mannitol, 2 mM Tris-Hepes, pH 7 4 and equilibrated at room temperature for at least one hour The uptake was determined in vesicles equilibrated with 88 5 mM NaCl and increasing concentrations of sucrose in the incubation medium for at least 30 min The tonicity of the medium with respect to non-penetrating, non-electrolytes (sucrose) was expressed as relative osmolality where 1 = isosmolar = 300 mosM Values shown represent total uptake at 15 s measured after incubation with 0 13 mM L-[^{35}S]cystine (0 2 μCi) together with 0 6 μCi of L-[^{3}H]glucose at 22°C, and are the means ± S E for four determinations The line was drawn by the least-squares method Fig 1A shows the uptake of 0 13 mM L-[^{35}S]cystine and Fig 1B shows the uptake of L-[^{3}H]glucose in the presence of 0 13 mM L-cystine in response to medium osmolality changes

binding of cystine after membrane exposure to papain The experimental design was similar to that of McNamara et al [7] with variation in

medium sucrose to alter osmolality In these studies, 88 5 mM NaCl was equilibrated across the membrane and was not included in calculation of relative osmolality Fig 1A shows that the 15 s uptake of 0 13 mM L-cystine by both control and papain-treated membranes was inversely proportional to the relative osmolality Extrapolation of the line for control vesicles to an infinitely high osmolality (where it intersects the ordinate and there is theoretically no intravesicular volume) indicates that 60% of the total uptake of L-cystine consisted of adsorption to the membranes under sodium equilibrated conditions These results are almost identical to those reported earlier [7,10] For papain-treated vesicles extrapolation of the line to the ordinate representing cystine uptake shows an intercept very close to the origin and indicates only about 8% of the total uptake was due to binding to the membranes

Fig 1B illustrates that papain treated vesicles are not altered in their osmotic reactivity The uptake of tracer L-[^{3}H]glucose by papain-treated vesicles with increasing osmolality decreases along the same line as that found in control vesicles Extrapolation of the line to origin is consistent with the known properties of L-glucose entry into vesicle space by diffusion and its lack of any membrane adsorptive component [11]

Effect of papain on the uptake of L-cystine

Papain treatment of membrane proteins was carried out by incubating brush-borders at 37°C for 10 min in buffer consisting of 0 25 M sucrose, 0 01 M triethanolamine hydrochloride (pH 7 6) as previously described [4] Uptake by these vesicles measured after spinning, washing and resuspension in buffer A is shown in Table I Under sodium gradient conditions a marked increase in total uptake (bound plus free) of cystine at 15 s is observed with all of the papain concentrations employed In the same membrane preparations (Table I) papain treatment caused the opposite effect on uptake of proline, a diminution which has been extensively studied in our earlier report [4] Since the latter work was performed with 0 015 mg activated papain per ml of membranes preparation, this concentration was selected as the standard for further studies on cystine transport Such treatment removed an insignificant amount

TABLE I

EFFECT OF PAPAIN CONCENTRATION UPON THE UPTAKE OF L-CYSTINE AND L-PROLINE UNDER Na^+-GRADIENT CONDITIONS

The brush-border vesicles were incubated at 37°C for 10 min in the absence or presence of 0 005 mg, 0 015 mg, 0 045 mg activated papain per ml of membrane preparation Control and papain-treated vesicles were centrifuged and washed at the end of incubation as described in the text Membrane fractions were suspended in 100 mM mannitol, 2 mM Tris-Hepes (pH 7 4) and equilibrated at room temperature for at least one hour The uptake of 0 03 mM L-[^{35}S]cystine and 0 02 mM L-[^{14}C]proline was determined in vesicles under an inwardly directed 88 5 mM NaCl gradient For L-cystine uptake, 50 μl of vesicle suspension was added to medium containing 15 μl of [^{35}S]cystine mix in H_2O (including 5 μl of 0 5 M HCl to initially dissolve the stock L-cystine and 0 2 μCi of L-[^{35}S]cystine) and 0 5 ml of buffer (60 mM mannitol, 20 mM Tris-Hepes, 100 mM NaCl (pH 7 6)) The pH of the final incubation mixture was 7 4 For L-proline uptake, 50 μl of vesicle suspension was added to medium containing 15 μl of [^{14}C]proline mix in H_2O (including 0 2 μCi of [^{14}C]proline tracer) and 0 5 ml of the same buffer used for vesicle suspension containing 100 mM NaCl Total uptake was measured at the end of 15 s incubation at 22°C Values represent the mean ± S E of four determinations

Papain concn (mg/ml)	Total uptake (nmol/mg)	
	L-[^{35}S]cystine	L-[^{14}C]proline
0	0 067 ± 0 003	0 542 ± 0 020
0 005	0 194 ± 0 016	0 191 ± 0 004
0 015	0 254 ± 0 066	0 296 ± 0 013
0 045	0 210 ± 0 010	0 212 ± 0 009

(about 2%) of total membrane protein, 85% of L-leucine aminopeptidase, 60% of gamma-glutamyltranspeptidase and 96% of alpha-D-glucosidase but did not effect the fragility of the vesicles nor their osmotic responses [4]

The time-course of 0 13 mM cystine uptake under sodium gradient conditions is shown in Fig 2 No 'overshoot' was observed with either control vesicles as reported previously [7] or with papain-treated vesicles Total uptake by papain-treated vesicles was higher during the initial two minutes of incubation At the end of 10 min of incubation, however, the level of uptake was the same in the control and papain-treated vesicles The increase in uptake during the first two minutes appears to occur during the 2 min interval it takes sodium to equilibrate in papain

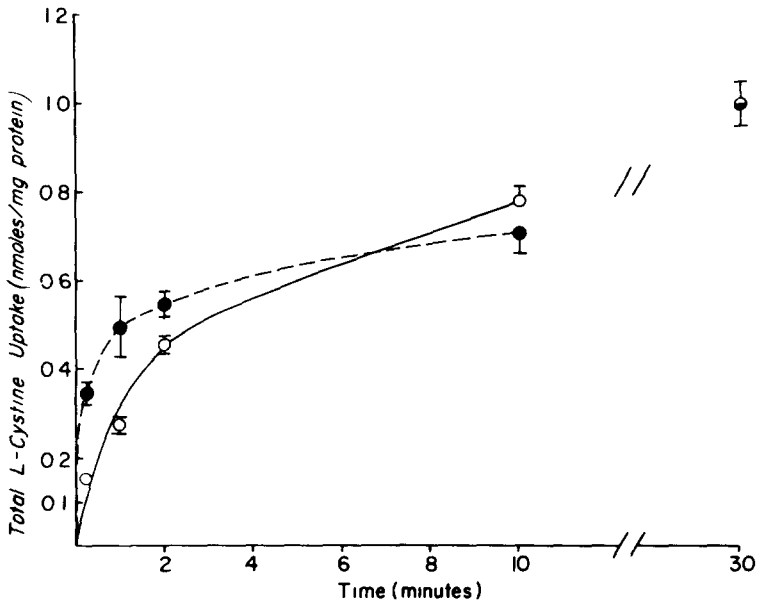

Fig 2 Effect of papain on the time-course of total uptake of L-cystine under a Na^+-gradient Control and papain-treated vesicles were obtained as described in the legend of Fig 1 The time-course of uptake by control (○) and papain-treated (●) vesicles was measured under the condition of 88 5 mM inward Na^+-gradients at 22°C in the presence of 0 13 mM L-[^{35}S]cystine (0 2 μCi) Values shown are the mean ± S E for four determinations Data points without brackets indicate that the S E is included within the point

treated vesicles as compared to the 10 min interval it takes sodium to equilibrate in control membranes [4]

Table II shows the data obtained when the uptake of 0 13 mM cystine was examined at 15 s under both sodium gradient and sodium equilibrated conditions No effect of papain treatment is observed when sodium is equilibrated across the membrane

TABLE II

EFFECT OF PAPAIN ON CYSTINE UPTAKE UNDER Na^+ GRADIENT VERSUS Na^+-EQUILIBRATED CONDITIONS

Brush-border vesicles were incubated at 37°C for 10 min in the absence or presence of 0 015 mg activated papain per ml of membrane preparation Control and papain-treated vesicles were obtained as described in the text The uptake of 0 13 mM were determined in vesicles under an inwardly directed 88 5 mM NaCl gradient and when 88 5 mM NaCl is equilibrated across the membrane as described in the legend for Table I Total uptake were measured at the end of a 15 s incubation at 22°C The number of determinations are indicated in the parentheses after the mean value ± S E

Incubation conditions	L-[^{35}S]cystine uptake (nmol/mg)	
	control vesicles	papain-treated vesicles
Na^+-equilibrated	0 133 ± 0 004 (13)	0 143 ± 0 007 (12)
Na^+-gradient	0 162 ± 0 014 (8)	0 320 ± 0 022 (8) *

* $P < 0\,001$ for difference from uptake of control vesicles

Influence of monovalent ions and the species of L-cystine

The study shown in Table III was performed to examine whether there is a greater enhancement in cystine uptake by papain-treated vesicles when they are incubated in the absence of sodium and whether the nature of the anion of the salt gradi-

TABLE III

EFFECT OF IONIC ALTERATION ON L-[^{35}S]CYSTINE UPTAKE

Membranes were prepared in 260 mM mannitol, 20 mM Tris-Hepes (pH 7 4) The incubation medium contained (final concentrate) 60 mM mannitol, 20 mM Tris-Hepes, 100 mM monovalent cation salts as indicated in the table Values given are mean ± S E for four determinations Net uptake value of L-cystine by control vesicles under inwardly direct 100 mM KCl gradient is 0 033 nmol/mg per 15 s which is taken as 100%

Ion gradient (100 mM)	% of 0 03 mM L-[^{35}S]cystine net uptake	
	control vesicles	papain-treated vesicles
Choline chloride	101 ± 6	96 ± 15
NaCl	175 ± 2 *	388 ± 10 **
Sodium gluconate	194 ± 8 **	264 ± 9 *

* $P < 0\,01$ for difference from uptake of control vesicles under 100 mM KCl gradient

** $P < 0\,001$ for difference from uptake of control vesicles under 100 mM KCl gradient

ent is a factor in the uptake process [15] In this experiment, the net uptake of cystine was determined, that is the free vesicular cystine present after membrane precipitation with trichloroacetic acid This was done to eliminate any differences in cystine uptake that could be due to changes in the binding component as a result of papain treatment although this component is not large at 15 s under sodium gradient conditions [7] The data in Table III show that there is no difference in cystine uptake between control and papain-treated vesicles when choline chloride forms the ion gradient and these do not differ from uptake with a 100 mM KCl gradient In papain-treated vesicles, uptake is stimulated 3-fold under Na^+-gradient conditions while in control vesicles uptake increases by 75% These findings in the absence of sodium are similar to those of Table II, where the baseline for comparison is the uptake when sodium is equilibrated across the membrane Thus, it clearly demonstrates that papain treatment stimulates sodium-dependent cystine uptake, while it does not affect the sodium-independent cystine uptake Also, the data in Table III indicate that the exaggerated uptake in papain-treated vesicles is not altered when chloride, a relatively fast diffusing anion, is replaced by gluconate, a relatively impermeant anion which suggested that cystine uptake is not altered by changes in membrane potential

Table IV shows that similar effects of papain upon L-cystine uptake were observed when medium pH was 5 5 and 8 0 as that was seen in medium pH 7 4 Bannai [14] has reported that at pH 5 5, L-cystine is entirely in the electroneutral form, and at pH 8 0, 55% of L-cystine is in anionic forms together with 45% of electroneutral L-cystine At medium pH 7 4, 30% of L-cystine is in the anionic form while the remaining 70% of L-cystine is in the electroneutral form Thus, the stimulation of the sodium-gradient dependent L-cystine uptake resulting from papain treatment occurred when cystine in the pH 5 5 medium was entirely in the electroneutral form

Effect of papain on membrane potential sensitivity of L-cystine transport

An effect of the membrane potential on the L-cystine uptake by control and papain-treated vesicles was tested in the absence of a Na^+-gradient by applying a potassium/valinomycin induced membrane diffusion potential as described by Jean et al [12] Vesicles were preloaded with 100 mM KCl, and L-cystine net uptake was studied in the presence of 20 μM valinomycin, a potassium specific ionophore, whose addition results in an inside negative membrane potential Fig 3 shows that neither L-cystine uptake by control vesicles nor that by papain-treated vesicles was sensitive to changes in membrane potential Also, preloading with potassium did not stimulate L-cystine uptake significantly compared with non-preloaded controls (data not shown) Fig 3 also

TABLE IV

EFFECT OF MEDIUM pH AND IONIC SPECIES ON L-CYSTINE UPTAKE

Membrane vesicles were washed, then pre-equilibrated for 1 h at 22°C in 200 mM mannitol buffered with 50 mM Hepes-Tris (pH 8 3) Total uptake of L-[^{35}S]cystine and trichloroacetic acid-precipitable membrane-bound L-[^{35}S]cystine were measured at the end of 15 s incubation by adding vesicle suspension to medium containing (final concentration) 0 03 mM L-[^{35}S]cystine, 100 mM NaCl, and 50 mM Hepes-Tris (pH 8 0), or 50 mM Mes-Tris, (pH 5 5) Net uptake values were obtained by subtracting trichloroacetic acid precipitable L-[^{35}S]cystine measurements from that of total uptake values Values represent mean ± S E of four determinations

Medium pH	Cystine ionic species [a]		Uptake of L-[^{35}S]cystine (nmol/mg protein per 15 s)	
			Control vesicles	papain-treated vesicles
8 0	$(^-OOC)_2R(NH_3^+)_2$	45%	0 055 ± 0 013	0 082 ± 0 008
	$(^-OOC)_2R(NH_2)NH_3^+$	45%		
	$(^-OOC)_2R(NH_2)_2$	10%		
5 5	$(^-OOC)_2R(NH_3^+)_2$	100%	0 107 ± 0 006	0 192 ± 0 002

[a] From Bannai, S [14]

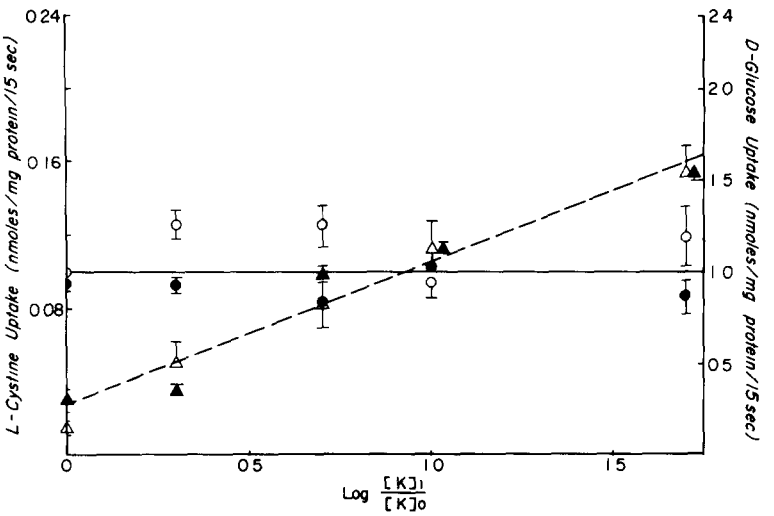

Fig 3 Effect of the membrane potential in the uptake of L-cystine by control and papain treated vesicles Control and papain treated vesicles were obtained as described in legend of Fig 1 Net uptake of L-cystine (○, ●) and D-glucose (△, ▲) by control vesicles (○, △) and papain-treated vesicles (●, ▲) Vesicles were preloaded with 100 mM mannitol, 20 mM Hepes-Tris (pH 7 4) 100 mM KCl and 10 mM NaCl Uptake were measured after 15 s in an incubation medium containing 100 mM mannitol, 20 mM Hepes-Tris (pH 7 4), 10 mM NaCl, 0 03 mM L-[^{35}S]cystine or 0 4 mM D-[^{14}C]glucose, 20 μM valinomycin and KCl to get the indicated ratio $[K^+]_i/[K^+]_o$ Choline chloride was added to the incubation medium to keep the osmolality constant

shows as a control the response of 0 4 mM D-[^{14}C]glucose uptake to the change in membrane potential The data which show glucose uptake by brushborder membrane to be membrane potential dependent is in full agreement with the observations of Jean et al [12] Papain treatment did not alter this dependence

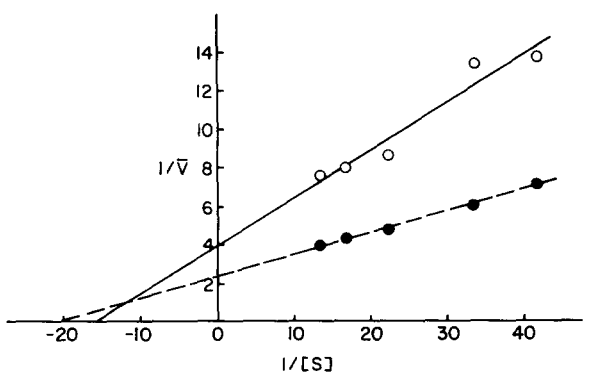

Fig 4 Lineweaver-Burk analysis of L-cystine uptake by brush-border vesicles Membrane fractions were obtained and described in the legend of Fig 1 Membrane fractions were suspended in 260 mM mannitol, 20 mM Hepe-Tris (pH 7 4) Uptake of control (○) and papain-treated (●) vesicles after 15 s of incubation at 22°C with a 100 mM NaCl- inward gradient containing 60 mM mannitol and 20 mM Hepes-Tris, was measured over a range of 0 024 mM to 0 075 mM L-cystine The velocity of net uptake, V, was measured as nmol/mg protein per 15 s, and [S] is the cystine concentration in the incubation medium The lines represent the best fit for the data by using linear regression analysis Each datum point represents the mean for eight determinations

Effect of papain upon the kinetic parameters of L-cystine transport systems

The kinetics of L-cystine uptake have been measured from 0 024 mM to 0 075 mM in the presence of 100 mM NaCl inwardly directed gradient The net or free cystine uptake value at 15 s in each medium concentration studied was plotted according to Lineweaver-Burk method as shown in Fig 4 In the concentration range studied, only one transport system is predominant for both control vesicles and papain-treated vesicles i e the low K_m, high affinity system shared by dibasic amino acids as reported by Segal et al [13] with a K_m of 0 065 mM and V_{max} of 0 254 nmol/mg per 15 s for control vesicles For papain-treated vesicles, K_m of 0 049 mM and V_{max} of 0 414 nmol/mg per 15 s were obtained by Lineweaver-Burk analysis The data suggest that papain treatment may decrease the K_m but the predominant effect appears to be an increase in the V_{max} for cystine uptake If the ratio V_{max}/K_m is calculated as a measure of the efficiency of the process, the value of 4 for the control vesicles increases to 9 for papain-treated membranes

Discussion

In contrast to the diminished sodium gradient stimulated entry of proline and other amino acids into rat renal brush-border membrane vesicles exposed to papain [4] the rate of cystine uptake is

enhanced This difference between the response of the cystine transport system and that for proline to treatment of the membrane with papain adds to others already known, the fact that cystine uptake is stimulated by a sodium gradient without an 'overshoot' and that cystine binds to the vesicle membrane as the incubation time increases Both of these phenomena are influenced by membrane papain treatment

In response to the removal of major marker enzyme activities but of only 2% or less of protein from the membrane by papain there is no change in the behavior of the vesicles to increasing osmolality Therefore, an alteration in unspecific permeability does not account for enhanced cystine uptake The mechanism of the papain effect is clearly related to the movement of sodium ion There is no enhanced vesicular cystine uptake after papain treatment when sodium is equilibrated across the membrane, only when a sodium gradient is imposed (Table II) The lack of a difference in uptake under sodium equilibrated conditions implies also that there is no effect of papain on the 'carrier' mechanism itself The importance of sodium ion in the papain effect is also emphasized by the data in Table III which shows that there is no enhancement of uptake when the incubation medium contains only choline chloride Thus, any sodium-independent cystine transport is unaffected by papain

Our previous observations that papain treatment decreased the sodium gradient stimulated 'overshoot' of proline and the uptake of several other amino acids led to experiments which showed that the entry of sodium into papain-exposed vesicles was five times faster than that to native vesicles For those amino acids where the energy for the overshoot is derived from the sodium gradient, a more rapid dissipation of the gradient is consistent with a decrease in the overshoot Since cystine entry in control vesicles under a sodium gradient does not occur with an overshoot but with a more rapid achievement of equilibrium than in the absence of a sodium gradient, the mechanism of the sodium gradient stimulated uptake could be assumed to be different from proline and other amino acids [16,17] The data of the present experiments suggest that this is the case and that cystine uptake is more closely coupled to the movement of sodium into the vesicle rather than to the energy derived from the gradient This assumption is supported by the fact that the increase in cystine uptake by papain-treated vesicles lasts about two minutes which is the time that Na^+ takes to equilibrate across the treated membrane The faster influx of cystine with the rapid entry of sodium is reflected by the 65% increase in the V_{max} of cystine entry whereas the V_{max} for proline uptake which is gradient dependent is decreased

We have explored the possibility that cystine uptake was electrogenic in nature and that papain could affect this property of cystine transport The data indicate cystine transport not to be electrogenic and that papain does not alter this characteristic (Fig 3) Our conclusion regarding the electrogenic nature of cystine entry differs from that of Biber et al [10] who used the EGTA/magnesium precipitation method to isolate the membrane vesicles [18] On the basis of their data, it was concluded that cystine transport is electrogenic The difference in the results may be explained by the different methods of isolating vesicles Murer et al [19] have observed an increase of transport rate for Na^+-H^+ exchange by membrane vesicles isolated by the EGTA/magnesium precipitation method when compared to that of magnesium precipitation method alone It may be explained also by the difference in the incubation systems which the two laboratories used to study the effect of potassium-valinomycin induced diffusion potential on L-cystine uptake Biber et al [10], in addition to the potassium-valinomycin induced potential difference on the two sides of the vesicular membranes, employed a 100 mM sodium gluconate inwardly directed gradient However, we did not impose a Na^+-gradient while measuring the effect of membrane potential upon the L-cystine uptake

Bannai [14] has emphasized that cystine can exist as several ionic species depending on pH Previous studies of cystine transport by renal brush-border membranes [7,10,13] as well as the observations of the effect of papain treatment were made at a physiological pH where cystine exists 70% in the electroneutral form and 30% with a negative charge [14] Whether a specific ionic species participates in the papain effect was ex-

amined by using medium pH of 5 5 where cystine exists entirely in an electroneutral form and at pH 8 0 where 55% exists as negative ionic species The enhanced sodium gradient stimulated entry observed after papain treatment of the membrane seen at pH 5 5 was of the same order of magnitude as that seen at pH 7 4 (Tables II–IV) since the papain effect is observed when cystine is in the electroneutral form it appears clear that it is not the anionic form of cystine which follows the faster Na^+ movement Whether an effect on the anionic forms occurs is not clear due to the continued presence of electroneutral cystine at pH 8 0, the highest pH we employed

Treatment of the membranes with papain causes a decrease in cystine binding to membranes and tends to make cystine behave like other amino acids which are present in the intra-vesicular space and not membrane bound [4] This binding which was described previously [7] and is relatively small under sodium-gradient conditions at 15 s of incubation increased with time The bound cystine could be freed by exposure to sulfhydryl reagents but the release was not immediate, suggesting the cystine was not bound to the outer membrane surface but associated with proteins in the interior or intravesicular face of the membrane [17] Apparently papain alters these cystine binding sites and affects them while leaving the cystine transporter interacting more efficiently with sodium ions These findings give credence to the interpretation that for the most part cystine binding is a secondary phenomena not related to the cystine transport process and probably occurs on the inner membrane surface after cystine has transversed the membrane The small amount of cystine binding remaining after papain treatment may reflect a more specific association with a cystine transporting protein It may well be that the elimination of the secondary binding may make the carrier mechanism more efficient and thus contribute to the enhanced uptake of free cystine into the intravesicular space The ability of papain to diminish binding may be important in future attempts to isolate specific cystine carrier proteins in the membranes if our assumption is correct that treatment with papain appears to eliminate non-specific cystine binding proteins Papain treated membranes may be a better starting point for purification and isolation of the proteins involved in the transport process than native membranes

Acknowledgement

This work was supported by Grant AM 10894 from the National Institutes of Health, Bethesda, MD

References

1 Rohn, R , Biber, J , Haase, W and Murer, H (1983) Mol Physiol 3, 3–18
2 Semenza, G , Kessler, M , Hosang, M , Weber, J and Schmidt, U (1984) Biochim Biophys Acta 779, 343–379
3 Hsu, B Y -L , Corcoran, S M , Marshall, C M and Segal, S (1982) Biochim Biophys Acta 689, 181–193
4 Hsu, B Y -L , Corcoran, S M , Marshall, C M and Segal, S (1983) Biochim Biophys Acta 735, 40–52
5 Booth, A G and Kenny, A J (1974) Biochem J 142, 575–581
6 Bradford, M M (1976) Anal Biochem 72, 248–254
7 McNamara, P D , Pepe, L M and Segal, S (1981) Biochem J 194, 443–3449
8 States, B , Lee, J and Segal, S (1983) Arch Biochem Biophys 220, 361–369
9 States, B and Segal, S (1969) Anal Biochem 27, 323–329
10 Biber, J , Stange, G , Stieger, B and Murer, H (1983) Pflugers Arch 396, 335–341
11 Hopfer, U , Nelson, K , Perrotto, J and Isselbacher, K (1973) J Biol Chem 248, 25–32
12 Jean, T Ripoche, P and Poujeol, P (1983) Membrane Biochem 5, 1–18
13 Segal, S , McNamara, P D and Pepe, L M (1977) Science 197, 169–171
14 Bannai, S (1984) Biochim Biophys Acta 119, 289–306
15 Levine R Hirayama, B and Wright, E M (1984) Biochim Biophys Acta 769, 508–510
16 McNamara, P D , Ozegovic, B , Pepe, L M and Segal, S (1976) Proc Natl Acad Sci USA 73, 4521–4525
17 McNamara, P D , Pepe, L M and Segal, S (1979) Biochim Biophys Acta 556, 151–2160
18 Biber, J , Stieger, B , Haase, W and Murer, H (1981) Biochim Biophys Acta 647, 169–176
19 Murer, H , Biber, J , Gmaj, P and Stieger, B (1984) Mol Physiol 6, 72