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Cystine uptake by rat renal brush-border vesicles

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Uptake of L-cystine by brush-border membrane vesicles isolated from rat renal-cortical tissue was time-dependent and occurred in the absence of cystine reduction. A significant capacity for vesicular binding of cystine was observed. The amount bound increased with time of incubation and could be displaced by thiol reagents. At early time points, cystine uptake measured the transport of cystine into the intravesicular space. Total cystine uptake was mediated by multiple transport systems, including a low-K_m high-affinity component which was shared by lysine, arginine, ornithine and glutamine and on which hetero-exchange diffusion of lysine and cystine was demonstrated.

The nature of the renal-tubule reabsorptive mechanism for L-cystine and the dibasic amino acids has been the subject of numerous investigations since cystinuria was first postulated to be a renal transport disorder (Dent & Rose, 1951; Robson & Rose, 1957; Webber *et al.*, 1961; Fox *et al.*, 1964). In our own laboratory, we have reported the characteristics of cystine transport by renal-cortical slices and isolated tubule fragments (Rosenberg *et al.*, 1962; Segal & Smith, 1969; Foreman *et al.*, 1980). We have also published a short communication on the interaction of cystine and dibasic amino acid uptake by brush-border membrane vesicles (Segal *et al.*, 1977). This preliminary work with isolated vesicles, however, did not delineate the nature of the membrane mechanism for cystine uptake. In the present report, we describe a more detailed investigation of the uptake process, including observations of Na^+ dependence, vesicular binding and hetero-exchange diffusion. In the light of these findings, we have also re-examined the concentration-dependent uptake of cystine under conditions that more closely approximate to the initial rate of uptake.

Materials and methods

Membrane preparation

Rat renal brush-border membrane vesicles were isolated from the kidneys of adult male Sprague–Dawley rats (Charles River, Wilmington, MA, U.S.A.) by the $MgCl_2$ precipitation method of Booth & Kenny (1974) modified as described previously (Weiss *et al.*, 1978). The final membrane preparation was suspended in THM buffer {100mM-mannitol in 2mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] which had been adjusted to pH 7.4 by the addition of Tris} to a final protein concentration of 2–3 mg/ml as determined by the method of Lowry *et al.* (1951) and by Bio-Rad protein determination (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Transport incubations

The standard uptake incubation consisted of 0.05 ml of brush-border vesicles in THM buffer which were added to a disposable test tube containing 50 μmol of NaCl, 5 μl of unlabelled amino acid of the desired concentration in 0.5 M-HCl, 0.1 μCi of ^{14}C-labelled amino acid, 0.2 μCi of ^{3}H-labelled 3-*O*-methyl-D-glucose or L-[^{3}H]glucose, and any other addition in water as desired, and 0.45 ml of buffer C (20mM-Tris + 20mM-Hepes + 60mM-mannitol). The final incubation was 100mM with respect to NaCl, with 0.2–0.3 mg of protein/ml in a final volume of 0.565 ml at pH 7.4. The measurement of uptake at 22°C was performed by using Millipore-filtration techniques published previously (McNamara *et al.*, 1976). Analysis of metabolic activity was performed by t.l.c. as described previously (Segal *et al.*, 1977), by using *N*-ethylmaleimide to prevent oxidation of thiol groups

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States & Segal, 1969). Uptake was measured as total membrane-associated radioactivity present on the filter with appropriate backgrounds subtracted. Backgrounds were determined by using heat-denatured vesicles or buffer in place of membrane vesicles.

The effect of osmotic perturbations on vesicle uptake of cystine and diffusible sugars was examined by adding sucrose to buffer C in the standard incubation in order to vary the osmolarity of the medium. The final incubation medium was 20 mM with respect to Tris, 20 mM-Hepes, 60 mM-mannitol and 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.

Exchange diffusion was measured by using membrane vesicles that had been preloaded with lysine by preincubating vesicles in THM buffer with 1 mM unlabelled lysine for 30 min before use in uptake experiments. Addition of 0.05 ml of the preincubation suspension to the standard incubation medium resulted in an 11-fold intravesicular/extravesicular concentration gradient of lysine. The effect of the lysine efflux on the influx of [^{14}C]cystine could then be examined.

To assess binding of [^{14}C]cystine to membrane vesicles, 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-fibre filters (no. 934AH; Reeve Angel, Clifton, NJ, U.S.A.) in a Millipore filter apparatus. The filtered precipitates were washed with 2 × 2 ml of 5% trichloroacetic acid and then with 2 × 2 ml of ethanol. Filters were air dried, placed in 4 ml of dilute Concentrol (Yorktown Research, Miami, FL, U.S.A.) in glass mini-vials and assayed for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer.

Materials

The following radiochemicals were obtained from New England Nuclear Corp., Boston, MA, U.S.A., and were chromatographically pure: L-[U-^{14}C]-cystine (296 mCi/mmol), L-[U-^{14}C]proline (248 mCi/mmol), [U-^{14}C]glycine (109 mCi/mmol), L-[U-^{14}C]glutamine (220 mCi/mmol), L-[U-^{14}C]-lysine (286 mCi/mmol), 3-*O*-[*methyl*-^{3}H]methyl-D-glucose (80.8 Ci/mmol), L-[1-^{3}H(n)]glucose (17.5 Ci/mmol) and L-[U-^{14}C]arginine (298 mCi/mmol). α-Methyl D-[U-^{14}C]glucoside (360 mCi/mmol) was purchased from Rose Chem Products, Los Angeles, CA, U.S.A. Unlabelled amino acids were from Mann Research Laboratories, New York, NY, U.S.A. Hepes buffer was obtained from Calbiochem, San Diego, CA, U.S.A.

Results

Uptake of cystine

The time course of uptake of L-[^{14}C]cystine under conditions of a NaCl gradient and when Na^+ was equilibrated across the membrane is shown in Fig. 1. Uptake values were higher in the presence of a Na^+ gradient than in the absence of the gradient for all time points earlier than 90 min; however, no 'overshoot' such as those characterizing the electrogenically stimulated transport of glucose (Kinne *et al.*, 1975; Beck & Sacktor, 1978) or other amino acids (McNamara *et al.*, 1976; Evers *et al.*, 1976) was observed at either low (0.03 mM) or high (0.66 mM) concentrations of cystine.

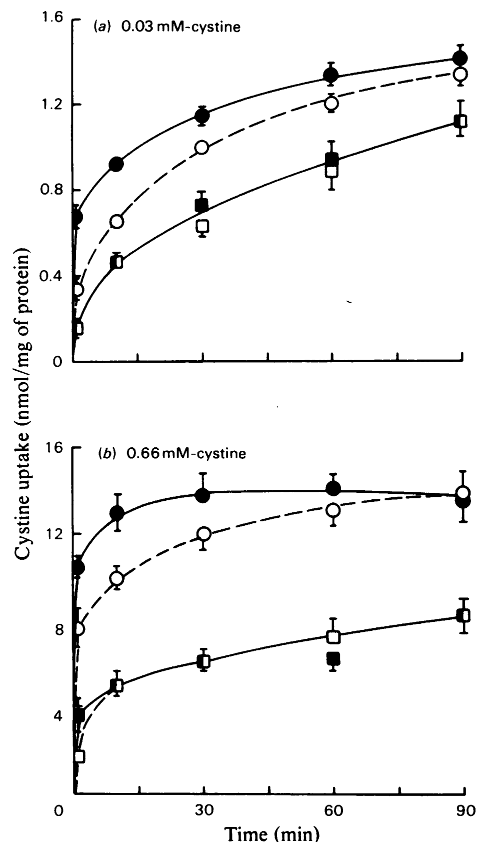

Fig. 1. *Uptake of (a) 0.03 mM- and (b) 0.66 mM-cystine by isolated rat renal brush-border membrane vesicles in the presence of a Na^+ gradient (●) or when Na^+ is equilibrated across the membrane (○)*

Membrane-bound cystine (■, □) was measured by trichloroacetic acid precipitation of standard incubations under Na^+-gradient (■) and Na^+-equilibrated (□) conditions. Values shown are the means for 8–12 determinations. Vertical bars indicate S.E.M.; where no bar appears, the S.E.M. is included within the symbol used to designate the mean.

In order to ascertain whether the uptake of cystine observed was primarily due to transport into the vesicles, the effect on cystine uptake of increasing the osmolarity of the medium by the addition of sucrose was studied (Fig. 2*a*). Initial (15 s) uptake of 0.13 mM-cystine was inversely proportional to the relative osmolarity (iso-osmolar = 1) of the medium. In these studies, 100 mM-NaCl was equilibrated across the membrane and was not included in the determination of relative osmolarity. Extrapolation of the line for cystine uptake to an infinitely high osmolarity (where it intersects the ordinate) indicates that the contribution of adsorption to total uptake was very high (66%) under conditions of Na^+ equilibration. The vesicles used could be shown to be osmotically reactive when 1 mM unlabelled cystine was present (Fig. 2*b*), since the initial uptake of 1 mM labelled L-glucose and 1 mM labelled 3-*O*-methyl-D-glucose decreased inversely with the osmolarity of the medium. Thus cystine itself did not alter the osmotic properties of the vesicles. The extrapolated line for uptake of L-glucose and 3-*O*-methyl-D-glucose passes through the origin, indicating that no binding component of membrane uptake exists for these diffusable sugars. All lines were determined by using the least-squares method on a Monroe 1775 programmable calculator.

Cystine binding to vesicles

In order to substantiate the indication of a large binding component suggested in the osmolarity studies, we have examined the amount of cystine binding to membrane protein by trichloroacetic acid precipitation. In these experiments, uptake in the standard incubation was stopped by the addition of trichloroacetic acid to a final concentration of 10%. The precipitated protein was then trapped on a glass-fibre filter and washed with 5% trichloroacetic acid and ethanol as previously described (McNamara *et al.*, 1974), and the amount of L-[^{14}C]cystine bound to the membrane protein determined. Controls where [^{14}C]cystine was added simultaneously with trichloroacetic acid showed that co-precipitation did not occur. The results in Fig. 1 show that a time-dependent component of uptake is present under both Na^+-gradient and Na^+-equilibrated conditions. Before 1 min of incubation, the binding component under Na^+-gradient conditions is small, with 74% of total uptake being soluble at 1 min. Although the amount of [^{14}C]cystine that was membrane-bound (acid-precipitable) was the same under the Na^+-gradient and Na^+-equilibrated conditions, total uptake was higher when measured under Na^+-gradient conditions than when Na^+ was equilibrated across the membrane. Thus the percentage of the total uptake that is bound under Na^+-equilibrated conditions seems much larger than in the presence of a Na^+ gradient. The trichloroacetic acid-soluble portion of total uptake appears to be free in the intravesicular space, since the result of subtracting the trichloroacetic acid-precipitable component from the uptake measured under changing osmotic conditions is a straight line which passes through the origin (Fig. 2).

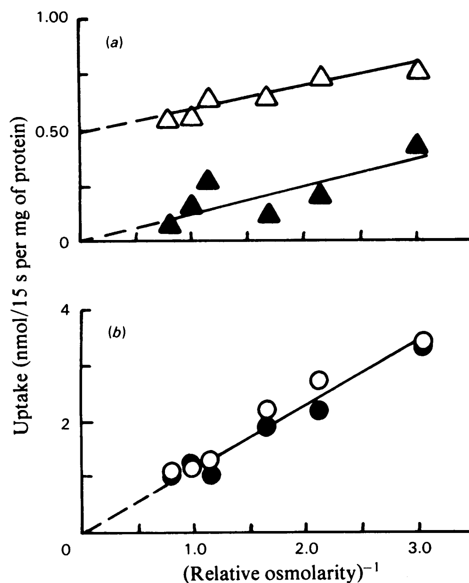

Fig. 2. *Effect of osmolarity on the uptake by brush-border vesicles of cystine (a) and of 3-O-methyl-D-glucose and L-glucose in the presence of 1 mM-cystine (b)*

(*a*) Vesicles were equilibrated with 100 mM-NaCl and increasing amounts of sucrose for 30 min before the addition of 0.13 mM-L-[^{14}C]cystine (△). Uptake was stopped by rapid filtration after 15 s of incubation at 22°C. The osmolarity of the medium with respect to non-penetrating non-electrolytes (sucrose and mannitol) was expressed as relative osmolarity, where iso-osmolar = 300 mosmol = 1.0, and 100 mosmol = 0.33. The inverse of relative osmolarity is plotted against uptake to determine the dependence of uptake on intravesicular space. The extrapolated point of intersection with the ordinate represents the adsorption component to uptake under Na^+-equilibrated conditions. Trichloroacetic acid precipitation was used to determine the amount of membrane-bound cystine, which was then subtracted from total cystine uptake. This soluble portion of cystine uptake was plotted similarly (▲). Values given represent the means for four determinations. (*b*) Vesicles were equilibrated as in Fig. 2(*a*) with NaCl and sucrose. In addition, 1 mM unlabelled cystine was present during the incubation. Uptake of 1 mM-L-[^{3}H]glucose (●) or 1 mM-3-*O*-[*methyl*-^{3}H]methyl-D-glucose (○) after 15 s of incubation was then measured as described above. All lines were drawn by using the least-squares method on a Monroe 1775 programmable calculator.

Membrane-associated L-[^{14}C]cystine is not easily removed when the vesicles are placed in cold water. However, 90% of the radioactivity trapped on the filter may be removed when the filter and membranes in water are heated for 6 min in a boiling-water bath. The ^{14}C-labelled substance removed from the membranes with this treatment was determined by t.l.c. of *N*-ethylmaleimide adducts (States & Segal, 1969) to consist almost exclusively of [^{14}C]cystine. Thus, no metabolic conversion of cystine into cysteine or any other compound was observed during normal incubations.

Further examinations into the nature of cystine binding were pursued by investigating the effect of cysteine and other thiol-containing or -protecting compounds on membrane-associated cystine. The results are presented in Table 1. For this, 2 mM-dithiothreitol, -reduced glutathione (GSH) or -cysteine was added to standard 60 min cystine incubations, resulting in a dramatic displacement over the next 10 min of incubation of the membrane-associated cystine that had accumulated during the first 60 min of incubation. The addition of an equal volume of water (5 μl) to a 60 min incubation (where 50% or more of uptake was membrane-bound) resulted in no diminution of accumulated cystine. Chromatographic analysis of incubation mixtures indicated that reduced glutathione is rapidly decomposed to its three component amino acids in the presence of brush-border protein. In vesicles pre-incubated with 0.03 mM- or 0.66 mM-cystine, membrane-associated cystine was displaced with equal rapidity by 2 mM-cysteine and 2 mM-dithiothreitol and less rapidly by 2 mM-glutathione. The fact that cysteine did not displace cystine from the membrane at the earliest time points measurable (⩽10 s) leads us to speculate that the [^{14}C]cystine bound to the membrane may be bound to the intravesicular face of the membrane, an interpretation suggested by Gmaj *et al.* (1979) in similar studies of Ca^{2+} uptake.

Concentration-dependence of uptake

The kinetics of total uptake for 15 s incubations under Na^+-gradient conditions, where the binding component is small, were examined and the results are seen in Fig. 3. Total cystine uptake appears to be mediated by two or more uptake systems, as evidenced by the two-limbed nature of the Hofstee plot for concentration-dependent uptake after 15 s of incubation. Membrane-bound cystine, as measured by trichloroacetic acid precipitation, represents a minor fraction of total 15 s uptake and is seen as a vertical line in the Hofstee plot in Fig. 3, showing no saturation kinetics. When the membrane-bound component is then subtracted from total uptake, the trichloroacetic acid-soluble uptake also presents a two-limbed Hofstee plot (Fig. 3), where K_m values of the two components are virtually unchanged from those of total uptake ($K_{m1} = 0.012$ mM and $K_{m2} = 6.6$ mM) and only the maximal velocities appear to be lowered slightly, especially for the high-K_m system (V_1 changes from 0.36 to 0.34 nmol/15 s per mg and V_2 decreases from 40.6 to 30.0 nmol/15 s per mg).

Interaction with other amino acids

To determine the specificity for cystine uptake, membrane vesicles were incubated with 0.03 mM-[^{14}C]cystine in the presence and absence of 1 mM unlabelled amino acids for 0.5 min, after which the incubation mixture was filtered and the uptake of cystine determined. The dibasic amino acids arginine, lysine, and ornithine, at 1 mM, inhibit uptake by 40–45%. Glutamine significantly inhibited 0.03 mM-

Table 1. *Displacement of cystine by thiol-containing reagents*

Standard transport incubations containing 0.03 mM- or 0.66 mM-cystine were performed for 60 min at 22°C in the presence of a Na^+ gradient. At 60 min of incubation, the mixtures were either filtered and assayed for control values of cystine uptake, or 5 μl of the compound specified was added to the mixture to yield a final concentration of 2 mM. The incubations were then filtered at the times designated after addition of the compounds and membrane-associated cystine was determined. Results are the means for four to eight determinations and are expressed as the percentage of the control 60 min uptake remaining at designated times after the additions.

Initial substrate concn.	Addition	Percentage remaining at designated time after addition			
		≤10 s	1 min	5 min	10 min
0.03 mM-cystine	Water	101.1	103.8	99.1	110.8
	2 mM-glutathione	104.2	83.35	60.4	53.3
	2 mM-cysteine	103.0	67.1	34.95	31.4
	2 mM-dithiothreitol	91.6	67.95	41.3	37.9
0.66 mM-cystine	Water	98.5	100.3	96.2	98.5
	2 mM-glutathione	102.1	87.5	64.2	56.6
	2 mM-cysteine	102.9	72.8	43.1	38.0
	2 mM-dithiothreitol	90.1	72.5	45.8	39.3

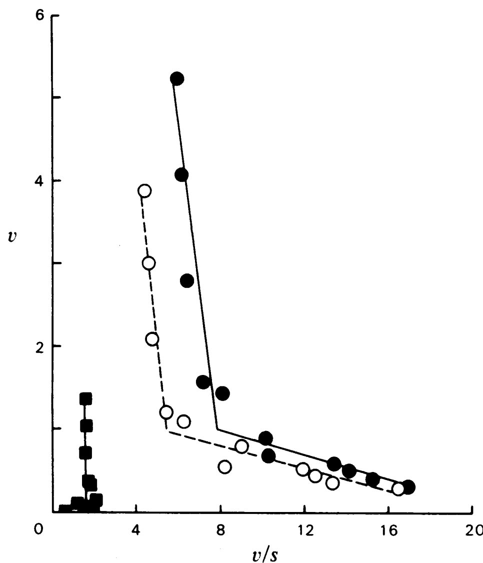

Fig. 3. *Concentration-dependence of initial (15 s) uptake and binding of cystine by brush-border membrane vesicles*
Vesicles were incubated with various concentrations of L-[^{14}C]cystine for 15 s at 22°C under Na^+-gradient conditions as described in the text. Total uptake (●) was measured and is shown as the plot of v versus v/s, where v is in nmol/15 s per mg of protein and s is in mM. Trichloroacetic acid was used to precipitate membrane-bound cystine (■). The soluble portion of cystine uptake was then calculated and is plotted (○). Values shown are means for eight determinations.

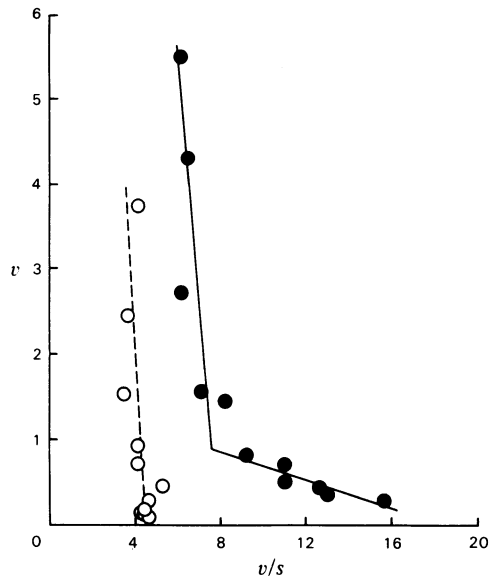

Fig. 4. *Influence of L-cystine concentration and of 1 mM-lysine on initial (15 s) uptake of cystine by brush-border membrane vesicles*
Vesicles were incubated with various concentrations of L-[^{14}C]cystine for 15 s at 22°C under Na^+-gradient conditions as described in the text in the presence (○) or absence (●) of 1 mM-lysine. Values represent the means for eight determinations.

cystine uptake by 18%. Phenylalanine, valine, glycine, proline and α-aminoisobutyric acid had no effect on cystine uptake. Thus it appears that low concentrations of cystine may be transported by an uptake system which is shared by the dibasic amino acids. Further evidence for this postulate is derived from the effect of 1 mM-lysine on the concentration-dependence of initial cystine uptake, which is shown in Fig. 4. Lysine acts as an inhibitor of the high-affinity system for cystine uptake, since only a single low-affinity system is evident when unlabelled lysine is present in the standard incubation.

Since uptake of low cystine concentration appears to occur via a transport system which is shared with dibasic amino acids, the phenomenon of hetero-exchange diffusion could be expected to occur. To examine this possibility, membrane vesicles in THM buffer were preloaded with 1 mM unlabelled lysine and used to measure the time course of uptake of 0.03 mM-[^{14}C]cystine under both Na^+-gradient and Na^+-equilibrated conditions. The results are shown in Fig. 5 as uptake relative to the 90 min point for Na^+-gradient conditions (Fig. 1). The presence of intravesicular lysine stimulates the initial uptake of extravesicular cystine in both the presence and absence of a Na^+ gradient, although the effect is greater (80% stimulation after 1 min) under Na^+-gradient conditions than in Na^+-equilibrium conditions (30% stimulation after 1 min). No difference in the trichloroacetic acid-precipitable fractions for cystine uptake by either lysine-loaded or control vesicles under Na^+-gradient or Na^+-equilibrated conditions was observed. Thus the phenomenon of hetero-exchange diffusion that we observed did not depend on the presence of the Na^+ gradient and had no effect on the intravesicular binding of transported cystine.

The effect of 1 mM-cystine on the initial uptake of proline, lysine, glycine, arginine and glutamine at 0.06 mM and on the uptake of the non-metabolizable sugar, α-methyl D-glucoside, was also examined. Of the substrates tested, only the uptake systems for 0.06 mM-lysine, -arginine and -glutamine are inhibited by 1 mM-cystine. The inhibition of dibasic amino acid uptake by cystine provides further proof for a shared system of uptake of these amino acids.

Discussion

We have shown that isolated rat renal brush-border membrane vesicles take up [^{14}C]cystine with

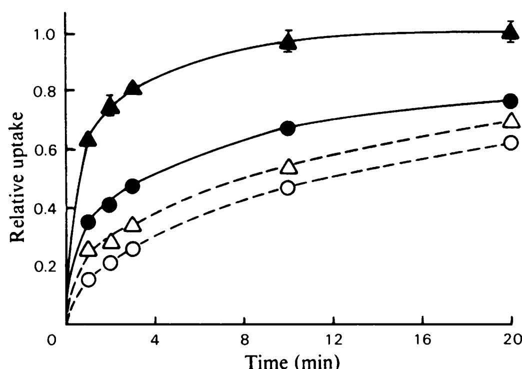

Fig. 5. *Effect of 1 mM-lysine efflux on influx of 0.03 mM-L-[14C]cystine under Na^+-gradient and Na^+-equilibrated conditions*

Vesicles in THM buffer were incubated with or without 1 mM-lysine and with or without NaCl for 30 min before being used in the standard incubation to measure cystine uptake. Total uptake was measured over a course of 20 min for all experimental conditions, and control uptake under Na^+-gradient conditions was determined for 90 min. The results are presented as relative uptake (90 min control = 1) for control vesicles under Na^+-gradient (●) and Na^+-equilibrated (○) conditions as well as for uptake for lysine-loaded vesicles under Na^+-gradient (▲) and Na^+-equilibrated (△) conditions. Results are the means for 20–36 determinations. S.E.M. values are included within the points unless indicated by bars.

a time-dependent course which shows no evidence of the standard overshoot phenomenon characteristic of Na^+-gradient dependence at either low or high concentrations of cystine. There is, however, a stimulation of uptake in the presence of a Na^+ gradient as compared with uptake when Na^+ is equilibrated across the membrane. The results of osmotic-perturbation studies performed under Na^+-equilibrated conditions indicate that a high degree of binding of cystine to the membrane occurs. This was confirmed by the precipitation of cystine–protein complexes with trichloroacetic acid. Membrane-bound cystine increases with time of incubation from 11% at 15 s to 75% after 90 min of incubation for 0.03 mM-cystine uptake under Na^+-gradient conditions. For 0.66 mM-cystine uptake under similar conditions, the trichloroacetic acid-precipitable fraction increases from 26% to 61% of total uptake.

The increasing percentage of bound cystine with time of incubation indicates a possibility that [14C]cystine may be transported into the vesicle and then bound. Studies done at initial (15 s) time points might indicate a slight degree of external binding, but the results of displacement studies performed when cystine uptake had reached a plateau indicate that thiol-containing compounds lower the amount of cystine in the vesicles only after being given time to penetrate the vesicles. At the shortest time intervals measurable (≤10 s), no such displacement occurred, leading us to speculate that the bound cystine might not be on the outer surface of the vesicle. The 11% of total 0.03 mM-cystine uptake that was trichloroacetic acid-precipitable at 15 s of incubation corresponds well to the value found for intact tubules (Foreman *et al.*, 1980).

A salient feature of the entry into intact cells is that cystine is reduced to cysteine (Segal & Smith, 1969; Foreman *et al.*, 1980) by an intracellular process involving reduced glutathione (Askelof *et al.*, 1974; Tietze *et al.*, 1972). This reduction does not occur in the isolated brush-border vesicles, our analytical procedures demonstrating the presence of cystine only in membrane vesicles that had been incubated with [14C]cystine. In isolated tubule cells, 90% of the cystine taken up by the cells is unbound even after 90 min of incubation, although as much as 75% is membrane-bound in isolated membrane vesicles. The continued reduction of cystine to cysteine by intact cells highlights our finding that thiol-containing compounds displace bound cystine from the membrane. This leads us to speculate that the coupling of the transport process to the reduction step in the intracellular environment at the membrane surface is a necessary event for the continued movement of cystine in the cell.

The uptake of cystine by isolated brush-border vesicles is mediated by at least two separate systems: a low-K_m high-affinity system, which appears to be shared with lysine, arginine, ornithine and glutamine, and a high-K_m low-affinity system, which appears to be unshared (Segal *et al.*, 1977). The two systems are reflected in the isolated tubule system (Foreman *et al.*, 1980), but the high-K_m system seems to be the only one observed with renal-cortical slices (Rosenberg *et al.*, 1962; Fox *et al.*, 1964). This indicates the need for a reinterpretation of the characteristics of cystine transport by utilizing the slice technique and a questioning of the results of cystine uptake by cortical slices from cystinuric patients (Fox *et al.*, 1964).

The characterization of two distinct processes for cystine uptake by brush-border membrane vesicles permits an interpretation of the clinical syndromes associated with abnormalities of renal-tubule handling of cystine and the dibasic amino acids. The postulate of Dent & Rose (1951) that classical cystinuria, with the characteristic urinary hyperexcretion of cystine, lysine, arginine and ornithine, is due to defective function of a shared transport system is supported by our data. Such a defect would reside in the low-K_m system. Hyperexcretion of cystine alone in a family reported by Brodehl *et al.* (1967) and observed in dog cystinuria (Bovée *et*

al., 1974) would result from a defect in the high-K_m system unshared by dibasic amino acids. The occurrence of kindreds with urinary loss of dibasic amino acids alone (Whelan & Scriver, 1968) indicates that a transport process specific for these amino acids is present in the kidney tubule and gives impetus for the evaluation of dibasic amino acid transport by brush-border membrane vesicles.

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