A Cysteine-specific Lysosomal Transport System Provides a Major Route for the Delivery of Thiol to Human Fibroblast Lysosomes: Possible Role in Supporting Lysosomal Proteolysis

Ronald L. Pisoni,* Tracy L. Acker,* Karen M. Lisowski,* Rosemary M. Lemons,* and Jess G. Thoene*‡

*Department of Pediatrics and Communicable Diseases and the ‡Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109-2029

Abstract. Lysosomes constitute only 4% of the intracellular volume of a normal human fibroblast. When human fibroblasts are incubated for 2–5 min with 20 μM [^{35}S]cystine in Krebs-Ringer phosphate solution at pH 7.4, a minimum of 50–60% of the total radioactivity taken up by the cells is found sequestered into the lysosomal compartment in the form of cysteine. A lysosomal transport system, highly specific for cysteine, appears to facilitate this rapid lysosomal cysteine sequestration. Time courses of [^{35}S]cysteine uptake into isolated, Percoll-purified fibroblast lysosomes at pH 7.0 and 37°C are linear for the first 4–5 min and attain a steady state by 10 min. Lysosomal cysteine uptake displays a K_m of 0.05 mM at pH 7.0 and an activation energy of 21 kcal/mol, corresponding to a Q_{10} of 3.2. The role of this transport system in delivering cysteine into lysosomes is supported by its pH curve showing a slow rate of cysteine transport at the acidic pHs between 5 and 6, but then increasing sevenfold between pH 6 and 7.5 to be maximally active near the cytosolic pH of 7. Carrier mediation by this lysosomal transport route demonstrates a high specificity for cysteine as indicated by the inability of the following amino acids to significantly inhibit at 5 mM the lysosomal uptake of 0.035 mM [^{35}S]L-cysteine: ala, ser, pro, val, gly, homocysteine, D- or L-penicillamine, arg, asp, or leu. Similarly, D-cysteine and β-mercaptopropionate were poor inhibitors, suggesting that both the L-isomer and α-amino group of cysteine appear to be required for recognition by the cysteine-specific transport system. In contrast, cysteamine, which lacks an α-carboxyl group, was able to strongly inhibit lysosomal cysteine uptake. The physiological importance of this cysteine-specific lysosomal transport system may be to aid lysosomal proteolysis by delivering cysteine into the lysosomal compartment to (*a*) maintain the catalytic activity of the thiol-dependent lysosomal enzymes and (*b*) break protein disulfide bridges at susceptible linkages, thereby allowing proteins to unfold, facilitating their degradation.

Lysosomes are a major intracellular site for the degradation of a wide variety of macromolecules which are delivered to this organelle by receptor-mediated endocytosis, pinocytosis, or autophagy. Many of the metabolites formed in the lysosome as a consequence of the breakdown of macromolecules appear to egress from the lysosomal compartment by way of carrier-mediated transport systems. Specific lysosomal transport systems for cystine (13–15, 19), the cationic amino acids (29, 31), small neutral amino acids (30), large neutral amino acids (6, 47), nucleosides (28), acidic monosaccharides (24, 32), and the sugars *N*-acetyl galactosamine and *N*-acetyl glucosamine (20) have been described. The importance of transport systems in mediating the escape of metabolites from the lysosomal compartment becomes especially obvious in the lethal genetic disease, nephropathic cystinosis. In this disorder, defective lysosomal cystine transport results in the lysosomal accumulation of cystine causing cystine crystals to form in various body tissues, with renal glomerular failure occurring by ten years of age (16). The lysosomal accumulations observed in two other inherited disorders, Salla disease and one form of methylmalonic aciduria, have been attributed to impaired lysosomal transport of sialic acid and cobalamin, respectively (32, 35).

In the present work we describe a lysosomal transport system highly specific for the amino acid cysteine. In contrast to many of the previously characterized lysosomal transport systems, this system appears to function for net delivery of its substrate into the lysosomal compartment rather than to serve for exodus of the products of lysosomal hydrolysis. This cysteine-specific transport route may play an important role in supporting lysosomal proteolysis by providing thiol for the lysosomal thiol-dependent proteases and by reducing protein disulfide bridges, thereby allowing proteins to unfold, facilitating their degradation.

A preliminary report of this work has appeared in abstract form (Pisoni, R. L., T. L. Acker, and J. G. Thoene. 1989. *Pediatr. Res.* 25:144A).

Materials and Methods

Cell Culture

Normal (GM0010A) and cystinotic (GM0090A) human fetal fibroblast cell lines were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts were grown and maintained in an atmosphere of 95% air, 5% CO_2 in 100-mm tissue culture dishes or 850-cm^2 roller bottles in Coon's modification of Ham's F-12 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (44). Human fibroblasts were routinely split 1:4 during passage and were not used beyond passage 22.

[^{35}S]Cystine Uptakes into Intact Human Fibroblasts

Cell culture medium was discarded from confluent 100-mm plates of fibroblasts, monolayers were washed twice with 10-ml portions of warm PBS, and fibroblasts were then incubated with 3 ml of a 0.02-mM [^{35}S]cystine solution ($\sim 2 \times 10^7$ cpm/plate) in sodium-containing Krebs-Ringer phosphate buffer, pH 7.4, supplemented with 0.1% glucose (8). The culture plates were gently rocked and, at the indicated times, incubations were terminated by aspirating the radioactive medium and washing the cell monolayers three times with 10-ml portions of ice-cold PBS. Cells were then scraped into 4 ml of ice-cold PBS containing 10 mM *N*-ethylmaleimide (NEM)[1] and collected by centrifugation at 750 *g* for 1.5 min at 4°C. Cell pellets were resuspended in 10 ml of 20-mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)/Tris (pH 7.0) buffer containing 0.275 M mannitol and 10 mM NEM and centrifuged at 750 *g* for 1.5 min at 4°C. The supernatant was discarded and the cell pellet resuspended in 3.5 ml of 10 mM MOPS/Tris (pH 7.6) buffer containing 0.25 M sucrose, 1 mM Na_2EDTA, and 10 mM NEM (lysis buffer). Cells were then lysed by up and down pipetting of the suspension 12 times as described previously (18, 29), and centrifuged at 750 *g* for 2.5 min at 4°C. The supernatants from three consecutive rounds of cell lysis were pooled and centrifuged at 1,500 *g* for 10 min at 4°C to remove nuclei. The resulting postnuclear supernatant, which contained >90% of the total radioactivity recovered from the cells, was centrifuged at 20,000 *g* for 15 min at 4°C in a rotor (SS-34; Sorvall Instruments Div., Newton, CT). Aliquots of the 20,000 *g* supernatant were assayed for radioactivity and hexosaminidase activity, whereas the 20,000 *g* pellet, which constitutes the granular fraction, was resuspended to 0.35 ml with the lysis buffer and aliquots counted for radioactivity and hexosaminidase activity.

The nature of the acid-soluble, intracellular radioactivity resulting from [^{35}S]cystine uptakes into intact fibroblasts was determined by adding 1/10 vol of 40% sulfosalicylic acid to the fraction, removing the precipitated proteins by centrifugation in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY) for 7 min at 4°C, and then subjecting the acid-soluble supernatant to either high voltage electrophoresis in 6% formic acid for 35 min at 3,500 V (44) or descending paper chromatography overnight in a solvent of *n*-butanol/acetic acid/H_2O (45:5:12.5). 30 nmol each of cystine, cysteine–NEM, and glutathione–NEM were included as internal standards with samples and were visualized with a ninhydrin spray. Sample lanes were then cut into segments of 1–2 cm in width and counted in 10 ml of Cytoscint scintillation fluid (ICN K & K Laboratories, Plainview, NY) to measure radioactivity.

[^{14}C]Alanine uptakes were performed as described for cystine uptakes, except that 2 mM aminooxyacetic acid was included in the incubation mixtures to inhibit transamination of alanine to pyruvate (10).

Preparation of Percoll-Purified Fibroblast Lysosomes

Fibroblast lysosomes were purified on Percoll density gradients as described previously (30), and were generally resuspended in 40 mM MOPS/Tris, (pH 7.0) buffer containing 0.25 M sucrose for use in most of the transport studies. Typically, 2–4 confluent roller bottles of fibroblasts were required per experiment to provide sufficient quantities of lysosomes for accurate measurement of lysosomal cysteine transport activity. The integrity of individual lysosomal preparations was generally found to be 80–90% intact as judged by the latency of β-*N*-acetylhexosaminidase activity, which was calculated as the difference in hexosaminidase activity in the presence or absence of 0.1% Triton. β-*N*-Acetylhexosaminidase activity was measured as described previously (28).

1. *Abbreviations used in this paper*: GSH, reduced glutathione; NEM, *N*-ethylmaleimide.

Lysosomal Cysteine Uptake Studies

Time courses at 37°C were initiated by mixing a prewarmed 200-μl aliquot of 0.108 mM [^{35}S]cysteine in 0.25 M sucrose containing 2 mM DTT with a 200-μl aliquot of lysosomes suspended in 40 mM MOPS/Tris (pH 7.0) buffer containing 0.25 M sucrose. At the indicated times, 26.5-μl aliquots were removed from the incubation mixture, added to 12 ml of ice-cold PBS, and filtered through a GF/A glass fiber filter (Whatman Inc., Clifton, NJ; 24 mm); the filter was washed twice with 12-ml portions of ice-cold PBS and then counted for radioactivity in 10 ml of Cytoscint scintillation fluid. Blanks for each experiment consisted of substituting 40 mM MOPS/Tris (pH 7.0) buffer containing 0.25 M sucrose for the lysosomes in the incubation mixtures. Aliquots removed from the blank incubations were filtered and washed in the same manner as for samples containing lysosomes. Radioactivity retained an the GF/A filters for these blank mixtures was subtracted from radioactivity retained on filters of lysosome-containing samples. 90% of the radioactivity taken up by fibroblast lysosomes during a 2.5-min incubation could be released from the lysosomes by exposing the lysosomal suspension to 0.1% Triton. In addition, uptakes were diminished 60% by increasing the osmolarity of the uptake incubation mixtures threefold. No significant metabolism of the [^{35}S]cysteine taken up by the lysosomes was observed as judged by high voltage electrophoresis.

For the K_m experiments, a 12-μl aliquot of 0.003 mM [^{35}S]cysteine in 10 mM MOPS/Tris (pH 7.0) buffer containing 0.25 M sucrose and 3 mM DTT was mixed with a 12-μl aliquot of unlabeled cysteine solutions of varying concentration in 40 mM MOPS/Tris (pH 7.0) buffer containing 0.25 M sucrose and DTT, with the DTT concentration being twice that of the unlabeled cysteine concentration. The tubes were warmed to 37°C and at time zero a 12-μl aliquot of ice-cold lysosomal suspension in 40 mM MOPS/Tris (pH 7.0) buffer containing 0.25 M sucrose was added to individual tubes and the mixtures incubated for 2.5 min at 37°C; a 27-μl aliquot was then removed from each incubation mixture, added to 12 ml of ice-cold PBS, lysosomes were collected on a GF/A glass fiber filter, washed, and counted for radioactivity as described above. The kinetic constants, K_m and V_{max}, and the nonsaturable component of uptake were calculated using the TOORGL program of Cleland (9) which applies the Gauss–Newton nonlinear least squares method in fitting the initial rate kinetic data to the following equation:

$$\log v = \log\left(\frac{V_{max} \cdot S}{K_m + S} + K_d \cdot S\right)$$

Analog inhibition experiments were performed in a manner similar to the K_m experiments except that the unlabeled cysteine substrate solution was replaced by an analog inhibitor solution containing DTT of a threefold greater molar concentration than the analog concentration.

Miscellaneous

[^{35}S]Cystine (400–500 Ci/mmol, 1 mCi/ml) was obtained from DuPont-NEN (Wilmington, DE) and was purified before use by diluting its specific activity to 80 Ci/mmol by the addition of unlabeled cystine, absorbing 250–500 μCi of radioactivity to 0.2–0.4 ml of Dowex 50W-X8 cation exchange resin (Bio-Rad Laboratories, Richmond, CA) in water, washing the resin five times with 10-ml portions of deionized water, and then eluting [^{35}S]cystine from the resin with 3 ml of 2 M NH_4OH. The eluted radioactivity was filtered through a 0.22-μm filter unit (Spin-X; Costar, Data Packaging Corp., Cambridge, MA), dried under nitrogen, and redissolved in 0.5 ml of distilled water for use. Radioactivity prepared in this manner migrated as a single spot with a cystine standard both by high voltage electrophoresis and paper chromatography. [^{35}S]Cysteine was prepared from the purified [^{35}S]cystine by the addition of DTT as indicated in the various experiments. Percoll was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), FBS was from Gibco Laboratories, *N*-ethylmaleimide was from Calbiochem-Behring Corp. (San Diego, CA), and amino acids were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

When intact normal human fibroblasts are incubated at 37°C with 0.02 mM [^{35}S]cystine for short intervals ranging from 2 to 5 min, the total acid-soluble radioactivity present in the cells is recovered mainly as cysteine with minor amounts of cystine also being observed (data not shown). Previous

studies have shown that this rapid metabolism of [^{35}S]cystine within the fibroblast is due to reduction of cystine by the high concentrations of reduced glutathione (GSH) present in the cytosol (27, 46). We examined if any of the radioactivity derived from the [^{35}S]cystine taken up by normal human fibroblasts during these short incubations is associated with lysosomes. Intact fibroblasts were incubated for 2 or 5 min with 0.02 mM [^{35}S]L-cystine at pH 7.4, cells were harvested and then subjected to subcellular fractionation in the presence of 10 mM NEM. The results shown in Table I indicate that ~50–60% of the total cystine radioactivity taken up by the fibroblasts was associated with the granular pellet; 99% of this radioactivity sequestered into the granular pellet was recovered as cysteine (Fig. 1).

When the granular pellet from the [^{35}S]cystine uptake experiments was fractionated on Percoll density gradients, the radioactivity associated with the granular pellet was distributed throughout the gradient in close correlation to the distribution of the lysosomal marker enzyme, β-*N*-acetylhexosaminidase (Fig. 2). Whereas mitochondria, Golgi vesicles, plasma membrane vesicles, and ER are known to sediment near the top of these Percoll gradients, the great majority of the radioactivity was localized to the bottom one-third of the Percoll gradient in which only densely sedimenting lysosomes are found (33). The association of this radioactivity with lysosomes was further confirmed by the observation that when lysosomes were specifically made buoyant by preloading them with amino acid methyl esters (41) the lysosomal marker enzymes and radioactivity both shifted to the top of the Percoll gradient in a proportionate manner (data not shown). These results indicate that although lysosomes comprise only 4% of the intracellular volume of normal human fibroblasts (11), they sequester 50–60% of the total [^{35}S]cystine radioactivity taken up by intact fibroblasts during 2–5-min incubations. This radioactivity sequestered into fibroblast lysosomes is in the form of cysteine. A very similar sequestration of radioactivity into the granular pellet was obtained when cystine uptakes were performed with cystinotic fibroblasts (data not shown). Based on the relative amounts of hexosaminidase activity in the 20,000 *g* supernatant and 20,000 *g* pellet (granular fraction), we estimate that ~20% of the lysosomes are broken during the subcellular fractionation steps leading to the preparation of the granular pellet. Thus, if no lysosomes had been broken in obtaining the granular fraction, we would expect to see 62–75% of the total [^{35}S]cystine-derived radioactivity taken up by the fibroblasts to be located in lysosomes.

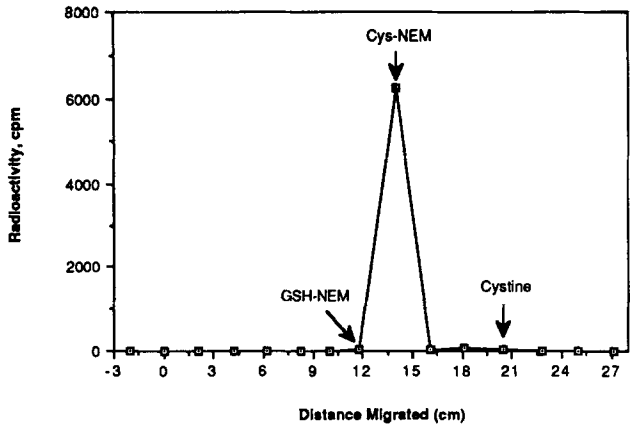

Figure 1. High voltage electrophoresis of the acid-soluble radioactivity present within the granular fraction of normal human fibroblasts following the uptake of 20 μM [^{35}S]cystine. Normal human fibroblasts were incubated with 20 μM [^{35}S]cysteine in Krebs-Ringer (pH 7.4) buffer at 37°C for 5 min; the cells were then washed three times with ice-cold PBS, lysed, and a granular fraction prepared as described in Materials and Methods. Proteins in the granular fraction were precipitated by the addition of sulfosalicylic acid, and an aliquot of the resulting acid-soluble radioactivity was subjected to high voltage electrophoresis as described in Materials and Methods. The position of the internal standards cystine, cysteine–NEM, and glutathione–NEM, relative to the distribution of radioactivity is shown.

Cystine is transported across the plasma membrane of human fibroblasts by the X^-_{CG} transport system (2–5). Substrates for the X^-_{CG} transport system at concentrations of 10 mM strongly inhibited the uptake of [^{35}S]cystine into fibroblasts resulting in a large decrease in the amount of radioactivity associated with the granular pellet (Fig. 3). In contrast, amino acids which are either not recognized or only weakly recognized by the X^-_{CG} transport system, had little effect on the amount of radioactivity sequestered into the granular pellet when these amino acids were added to the [^{35}S]cystine uptake mixtures. Therefore, the radioactivity associated with the granular pellet appears to be derived from [^{35}S]cystine transported into fibroblasts via the X^-_{CG} transport system.

We have previously described two lysosomal transport systems which recognize small neutral amino acids, lysosomal systems e and f (30). If [^{35}S]cysteine was being sequestered into fibroblast lysosomes from the cytosol by lysosomal systems e or f, then alanine, a known substrate for these two

Table I. Distribution of Radioactivity in Various Fibroblast Cell Fractions after a 2- or 5-min Cellular Uptake of 0.02 mM [^{35}S]Cystine or 0.02 mM [^{14}C]Alanine

	[^{14}C]Alanine uptakes		[^{35}S]Cystine uptakes	
	2 min	5 min	2 min	5 min
Lysate supernatants	36,259	39,715	35,010	83,083
1,500 *g* supernatant	36,738	40,813	29,250	72,527
20,000 *g* supernatant	33,843 (95%)	34,193 (85%)	14,535 (52%)	29,233 (43%)
20,000 *g* pellet	1,741 (5%)	5,818 (15%)	13,596 (48%)	38,342 (57%)

2 100-mm confluent plates of normal human fibroblasts were incubated with 3.0 ml of 0.02 mM [^{35}S]cystine or 0.02 mM [^{14}C]alanine in a Krebs-Ringer phosphate solution, pH 7.4, containing 2 mM aminooxyacetic acid for either 2 or 5 min at 37°C. At the end of this time, monolayers were washed three times with ice-cold PBS; cells were scraped from the plate into PBS containing 10 mM NEM, and collected by centrifugation; and the cell pellet was washed once with 20 mM MOPS/Tris (pH 7.0) buffer containing 0.275 M mannitol and 10 mM NEM. Cells were then lysed by up and down pipetting and a 20,000 *g* granular fraction prepared as described in Materials and Methods. The distribution of total radioactivity (cpm) recovered from the various cell fractions is listed.

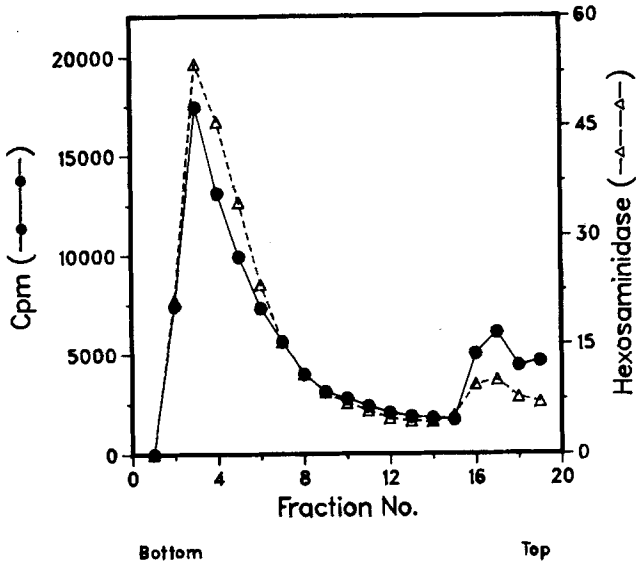

Figure 2. Subfractionation of the fibroblast granular fraction from [^{35}S]cysteine uptakes on Percoll density gradients. Normal human fibroblasts were incubated with 20 μM [^{35}S]cystine in Krebs-Ringer (pH 7.4) buffer for 5 min at 37°C, the cells were then washed three times with ice-cold PBS, and lysed; and a granular fraction was prepared as described in Materials and Methods. The granular fraction (0.35 ml) was placed on top of 10 ml of a 34% Percoll solution in 18 mM MOPS/Tris (pH 7.0) buffer containing 0.24 M mannitol and 1 mM Na_2EDTA, and centrifuged for 35 min at 17,500 rpm in a rotor (SS-34; Sorvall Instruments Div.) at 4°C. 0.5-ml fractions were collected from the bottom of the density gradient and assayed for hexosaminidase activity (△) and counted for radioactivity (●).

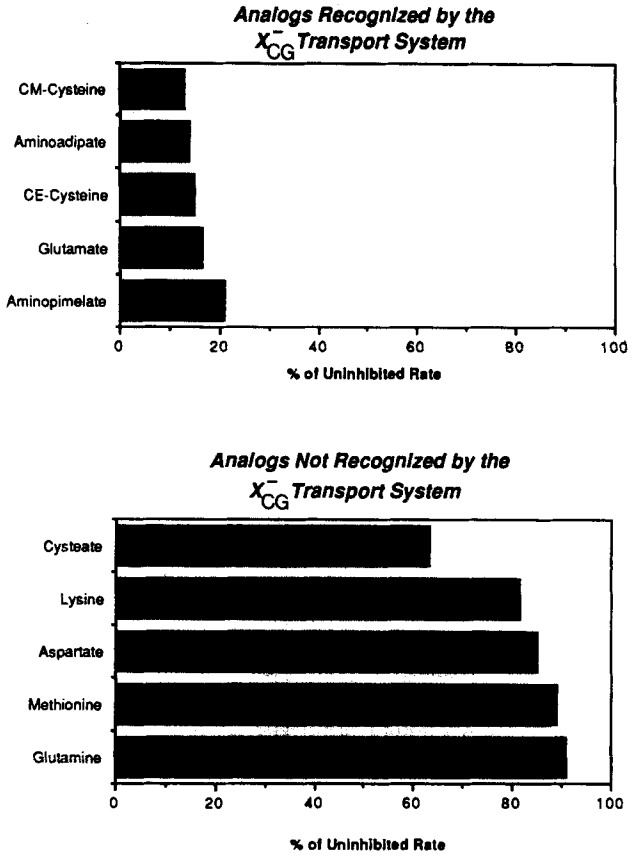

Figure 3. Ability of various amino acids, when added to the [^{35}S]cystine uptake assays, to cause a decrease in the amount of ^{35}S radioactivity associated with the granular fraction. Two confluent 100-mm plates of human fibroblasts were incubated with 20 μM [^{35}S]cystine for 5 min at 37°C in Krebs-Ringer (pH 7.4) buffer containing the indicated amino acid at a concentration of 10 mM. Incubations were terminated by washing the plates three times with ice-cold PBS, scraping the cells into PBS containing 10 mM NEM, and preparing a granular fraction as described in Materials and Methods. The amount of radioactivity present within the granular fraction per unit of hexosaminidase activity was determined for each amino acid listed and is given as a percent of the amount of ^{35}S radioactivity associated with the granular pellet per unit hexosaminidase activity in control incubations not exposed to analogs. *CM-Cysteine* and *CE-Cysteine*, Carboxymethyl- and Carboxyethyl-L-Cysteine, respectively.

transport systems, should also be found sequestered into fibroblast lysosomes when fibroblasts are incubated with alanine. To test this possibility, human fibroblasts were incubated with [^{14}C]alanine for 2 or 5 min and then the distribution of radioactivity among the various cell fractions was determined (Table I). In marked contrast to the results found with [^{35}S]cystine, only 5–15% of the total alanine radioactivity taken up by the fibroblasts was associated with the granular pellet. Furthermore, when the granular pellet from the [^{14}C]alanine uptakes was fractionated on Percoll density gradients, the radioactivity associated with the granular pellet did not sediment with the lysosomal marker enzyme, *β-N*-acetyl-hexosaminidase (Fig. 4). These results indicate that lysosomal transport systems e and f do not provide for any significant uptake of alanine into human fibroblast lysosomes during short uptake periods and that the process mediating lysosomal sequestration of cysteine does not recognize alanine and must be distinct from lysosomal transport systems e and f.

Isolated fibroblast lysosomes were then examined for the existence of a transport activity mediating this apparent lysosomal uptake of cysteine. At pH 7.0 and 37°C, [^{35}S]cysteine was observed to be taken up by fibroblast lysosomes in a linear fashion during the first 4–5 min of incubation and attained a steady state by 10 min (Fig. 5). DTT was included in the cysteine uptake incubation mixtures to prevent oxidation of cysteine to cystine. Replacing DTT by GSH had no effect on time courses of lysosomal cysteine uptake. Lysosomal cysteine uptake displayed a large temperature dependence, giving linear Arrhenius plots over the tested temperature range from 17 to 37°C from which an activation energy of 21 kcal/mol and a Q_{10} of 3.2 were calculated (data not shown). This is the highest activation energy found for any of the known lysosomal transport systems.

The potential role of this system in transporting cysteine into the lysosomal compartment from the cytosol is reflected by its pH curve. Very little transport of cysteine is observed at the acidic pHs between 5 and 6 but then transport increases 7–10-fold between pH 6 and 7.5 (Fig. 6). If this pH dependency exists on both sides of the lysosomal membrane, then once cysteine is transported into the lysosome from the cytosol, the intralysosomal pH of 5.3 (40) would not be favorable for exodus of cysteine from the lysosome by this transport route, thus resulting in net lysosomal cysteine ac-

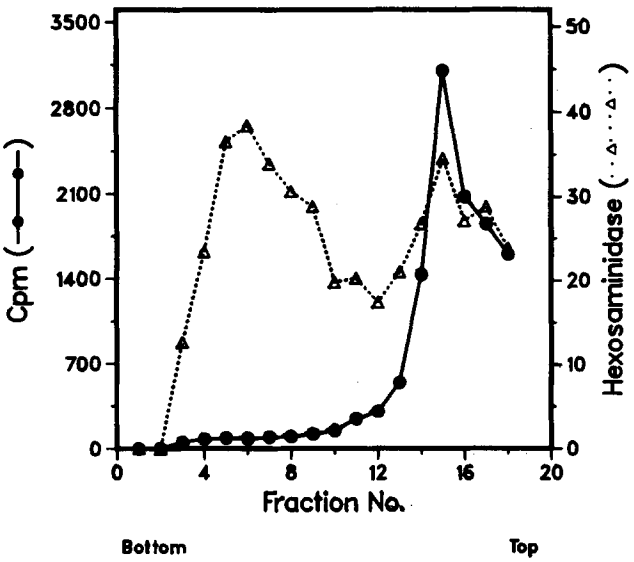

Figure 4. Subfractionation of the fibroblast granular fraction from [^{14}C]alanine uptakes on Percoll density gradients. Human fibroblasts were incubated with 20 μM [^{14}C]alanine in Krebs-Ringer (pH 7.4) buffer for 5 min at 37°C; the cells were then washed three times with ice-cold PBS, and lysed; and a granular fraction was prepared as described in Materials and Methods. The granular (0.35-ml) fraction was placed on top of 10 ml of a 34% Percoll solution in 18 mM MOPS/Tris (pH 7.0) buffer containing 0.24 M mannitol and 1 mM Na_2EDTA, and centrifuged for 35 min at 17,500 rpm in a rotor (SS-34; Sorvall Instruments Div.) at 4°C. 0.5-ml fractions were collected from the bottom of the Percoll density gradient and assayed for hexosaminidase activity (△) and counted for radioactivity (●).

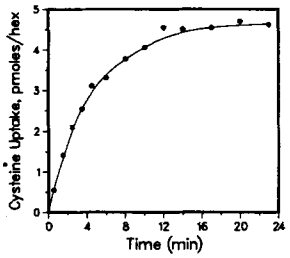

Figure 5. Time course of [^{35}S]L-cysteine uptake into Percoll-purified fibroblast lysosomes. Percoll-purified fibroblast lysosomes were incubated at 37°C with 0.054 mM [^{35}S]L-cysteine (1 Ci/mmol) in 20 mM MOPS/Tris (pH 7.0) buffer containing 0.25 M sucrose and 1 mM DTT. At the indicated time points, aliquots were removed from the incubation mixture and added to 12 ml of ice-cold PBS; lysosomes were then collected on GF/A filters, washed twice with 12-ml portions of ice-cold PBS, and the filters counted for radioactivity.

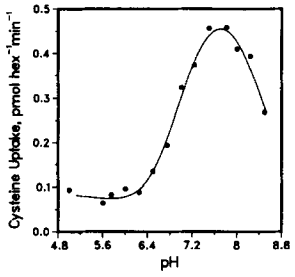

Figure 6. pH profile of 0.034 mM [^{35}S]L-cysteine uptake into fibroblast lysosomes. Fibroblast lysosomes (18 μl) suspended in 0.25 M sucrose were incubated with 18 μl of 0.068 mM [^{35}S]L-cysteine in either 15 mM 4-morpholineethanesulfonic acid (MES) or 15 mM MOPS buffers containing 0.25 M sucrose and 2 mM DTT which had been titrated to the indicated pH with Tris-free base. Incubations were performed at 37°C for 2.5 min at the end of which time 27-μl aliquots of the lysosomal incubations were filtered and washed, and the amount of radioactive cysteine taken up was counted. MES buffer was used from pH 5–6.5 and MOPS buffer from pH 6.5–8.5.

cumulation. We have not determined the basis of this pH dependency of lysosomal cysteine uptake. The initial rate of lysosomal cysteine uptake, however, is reduced by 60% when uptakes are performed at pH 7.0 in the presence of either 15 μM carbonyl cyanide *m*-chlorophenylhydrazone or 25 μM monensin containing 75 mM NaCl. These ionophores would be expected to dissipate the pH gradient across the lysosomal membrane. Though additional work is required in this regard, it is conceivable that lysosomal cysteine transport may be driven by protons in a manner analogous to that described by Mancini et al. (24) for the lysosomal sialic acid transport system.

A Michaelis–Menten plot (Fig. 7) of the initial rate of lysosomal cysteine uptake as a function of substrate concentration demonstrates that the uptake process is saturable and yields a linear Lineweaver–Burk plot (Fig. 7, *inset*). The apparent kinetic constants for lysosomal cysteine uptake at pH 7.0 and 37°C were calculated to be K_m = 53 μM ± 6 μM and V_{max} = 0.7 pmol min^{-1} hex^{-1} ± 0.04 using Cleland's TOORGL computer program (9). A small, nonsaturable component of uptake, K_d = 0.16 pmol min^{-1} hex^{-1} mM^{-1} ± 0.017, was accounted for by this computer analysis.

Analog inhibition experiments were performed to assess the substrate specificity of this lysosomal cysteine transport system (Table II). A high degree of specificity for cysteine is demonstrated by the inability of any of the amino acid analogs at concentrations of 5 mM to significantly inhibit lysosomal uptake of 0.035 mM [^{35}S]cysteine, whereas 5 mM L-cysteine was strongly inhibitory. Analogs very similar in structure to cysteine, such as homocysteine, penicillamine, and serine, demonstrated at best only a weak inhibition of lysosomal cysteine uptake. Furthermore, D-cysteine and the

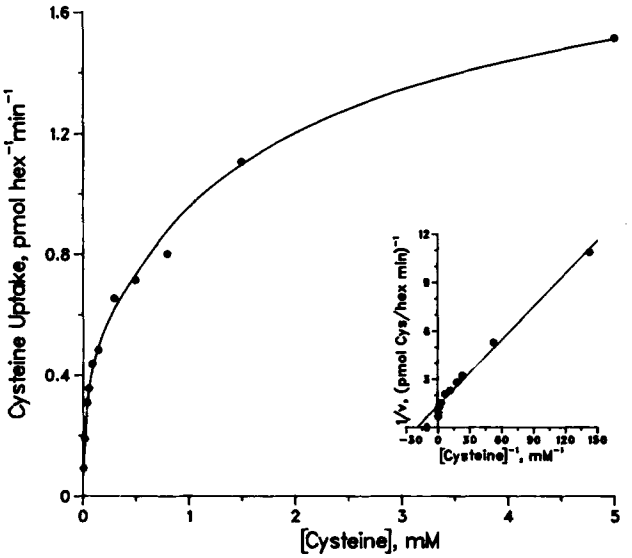

Figure 7. Kinetics of the initial rate of cysteine uptake into fibroblast lysosomes as a function of cysteine concentration. Fibroblast lysosomes were incubated for 2.5 min at 37°C with [^{35}S]L-cysteine of the indicated concentration in 30 mM MOPS/Tris (pH 7.0) buffer containing 0.25 M sucrose and DTT as indicated in Materials and Methods. At the completion of the incubation period, lysosomes were collected and washed on GF/A filters, and the amount of radioactive cysteine taken up was measured. A Michaelis–Menten plot of the initial rate of cysteine uptake as a function of the cysteine concentration is shown. (*Inset*) Double-reciprocal plot of the same data.

Table II. Inhibition of 0.035 mM [^{35}S]Cysteine Uptake into Fibroblast Lysosomes by Various Amino Acid Analogs

Amino Acid	% of Uninhibited Rate
L-Cysteine	6
Cysteamine	17
D,L-Homocysteine	80
D-Cysteine	83
L-Serine	84
L-Leucine	86
L-Penicillamine	89
L-Valine	90
L-Proline	97
β-Mercaptopropionate	98
L-Alanine	99
Glycine	101
L-Arginine	105
L-Aspartate	110
D-Penicillamine	119

The uptake of 0.035 mM [^{35}S]cysteine into fibroblast lysosomes was measured in the presence or absence of each of the listed amino acids each at a concentration of 5 mM, except D,L-homocysteine which was at 10 mM. Uptakes were performed for 2.5 min at 37°C in 30 mM MOPS/Tris (pH 7.0) buffer containing 0.25 M sucrose and 15 mM DTT (except 30 mM DTT was used in the case of homocysteine). At the completion of the incubation period, aliquots were removed from the given incubation mixture, lysosomes were collected and washed on GF/A filters, and filters were counted for radioactivity. The resulting amount of radioactivity taken up per analog tested is expressed as a percent of the uninhibited control.

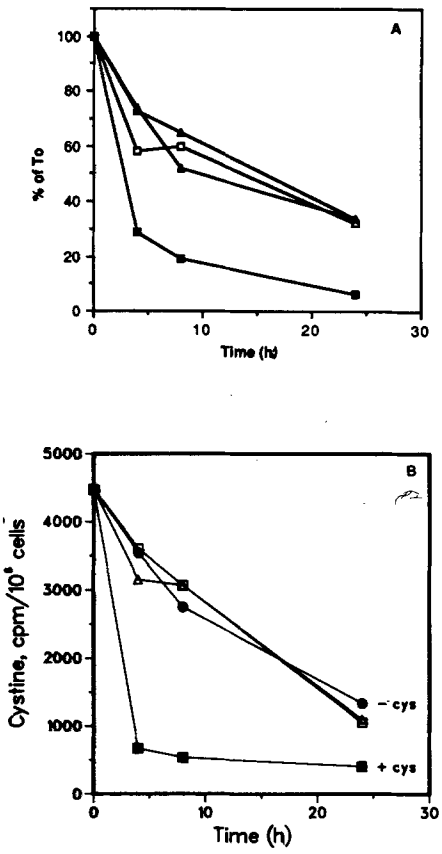

Figure 8. Loss of [^{35}S]cystine or [^{3}H]mannitol from cystinotic fibroblasts in cystine-containing vs. cystine-deficient culture media. (*A*) Confluent 60-mm plates of cystinotic fibroblasts were incubated for 24 h in 3 ml of cystine-deficient culture media supplemented with 10% dialyzed FCS containing either [^{3}H]mannitol (2.2×10^6 cpm/ml; 19 Ci/mmol) or [^{35}S]cystine (1.9×10^6 cpm/ml; 460 Ci/mmol). The plates were then washed three times with PBS and at time zero exposed to culture media deficient in cystine (*−cys*) or exposed to culture media containing 0.133 mM cystine (*+ cys*). At the times of 0, 4, 8, and 24 h, individual plates were harvested and the amount of [^{35}S]cystine or [^{3}H]mannitol radioactivity associated with the cells at these different time points is given as a percent of the time zero (T_0) cell-associated radioactivity: loss of [^{35}S]cystine from fibroblasts in cystine-containing (■) and cystine-deficient media (□); loss of [^{3}H]mannitol from fibroblasts in cystine-containing (▲) and cystine-deficient culture media (△). (*B*) Cystinotic fibroblasts were labeled for 24 h with [^{35}S]cystine (1×10^6 cpm/ml; 682 Ci/nmol) as described above, washed three times with PBS, and at time zero exposed to cystine-deficient culture media supplemented with either 0.1 mM cystathionine, 0.1 mM homocystine, 0.1 mM cystine, or no further addition. At the times of 0, 4, 8, and 24 h, individual plates were harvested and the amount of [^{35}S]cystine recovered from the cells at these different time points is given: loss of [^{35}S]cystine from fibroblasts exposed to cystine-deficient culture media only (●), or this same media supplemented with either 0.1 mM cystathionine (△), 0.1 mM homocystine (□), or 0.1 mM cystine (■).

deaminated form of cysteine, β-mercaptopropionate, had little if any effect on lysosomal [^{35}S]cysteine uptake indicating that both the L-isomer and the α-amino group of cysteine are important for recognition by the transport protein receptor site. The α-carboxy group of cysteine, however, does not appear to be required for recognition in that cysteamine was found to strongly inhibit lysosomal cysteine uptake.

All of the studies reported above were repeated with lysosomes from cystinotic fibroblasts, in which case the time course of lysosomal cysteine uptake, Arrhenius plot, pH curve, and substrate specificity were very similar to those found with lysosomes from normal human fibroblasts. Two small differences, however, were detected in the lysosomal cysteine transport activity of the cystinotic fibroblast cell line which was compared with the normal fibroblast cell line: (*a*) a K_m of 0.1 mM was found for lysosomal cysteine uptake by cystinotic lysosomes compared with 0.05 mM in normal lysosomes, and (*b*) lysosomal cysteine uptake was stimulated twofold when 4.5 mM MgATP was added to incubation mixtures of cystinotic lysosomes, whereas no stimulation was observed with normal fibroblast lysosomes.

Mannitol is a membrane-impermeant marker which accumulates in the lysosomal compartment as a result of pinocytosis, and is lost via exocytosis (22, 26). The rate of mannitol loss from cystinotic fibroblasts is the same ($t_{1/2} \cong 17$ h) whether cells are incubated in cystine-free or cystine-containing culture medium (Fig. 8 *A*). When cystinotic fibroblasts are prelabeled with [^{35}S]cystine for 24 h, free [^{35}S]cystine accumulates intracellularly and is exclusively localized within the lysosomal compartment (27, 36, 38). Upon being washed and placed in cystine-free culture medium, cystinotic fibroblasts lose [^{35}S]cystine at the same slow rate observed for lysosomal loss of [^{3}H]mannitol; i.e., at the exocytosis rate. When 0.1 mM unlabeled cystine is added to the culture medium, however, 85% of the intracellular [^{35}S]cystine is lost within the first 4 h of incubation. Although a rapid loss of intracellular [^{35}S]cystine is observed when cystinotic fibroblasts are incubated in cystine-containing culture medium, there is no loss in the total amount of intracellular free cystine indicating that this loss

of intracellular [^{35}S]cystine represents an exchange reaction. The basis for this exchange reaction is now apparent. When unlabeled cystine is transported into the cytosol it is reduced by GSH to form cysteine which then enters the lysosome by the cysteine-specific lysosomal transport system. Once within the lysosome, unlabeled cysteine exchanges with [^{35}S]cystine to form unlabeled cystine and [^{35}S]cysteine, with this latter product egressing from the lysosome by one of the transport routes capable of recognizing cysteine. In contrast to cystine, the analogs homocystine and cystathionine do not accelerate [^{35}S]cystine loss from cystinotic fibroblasts (Fig. 8 *B*). Upon entering the cytosol, homocystine would be expected to form homocysteine when reduced by GSH. Homocysteine, however, is not recognized by the cysteine-specific lysosomal transport system (Table II) and as a consequence homocysteine would not be able to enter lysosomes and exchange with [^{35}S]cystine. The net result is that homocystine added to cystine-free culture medium has no effect on the loss of lysosomal [^{35}S]cystine from cystinotic fibroblasts. Although we find that cystathionine is transported into fibroblasts at a rate similar to that for cystine (data not shown), it can not be reduced by GSH and is not able to participate in disulfide/sulfhydryl exchange reactions with cystine.

Discussion

The lysosome contains a large repertoire of enzymatic activities which function optimally at the acidic environment of this organelle to degrade a wide variety of macromolecules. Over the course of the last 10 years, various investigations have pointed to the role of thiol in stimulating the rate of lysosomal degradation of disulfide-rich proteins. In 1979, Griffiths and Lloyd (17) observed an enhanced rate of ^{125}I-insulin degradation by disrupted rat liver lysosomes when reduced glutathione was added to incubation mixtures. Later, Kooistra et al. (21) found that a variety of thiols were able to stimulate the rate at which purified lysosomal enzymes could degrade ^{125}I-labeled insulin or albumin. Among the lysosomal enzymes studied was cathepsin D, which is not a thiol-dependent enzyme. These authors postulated that the two processes of proteolysis and disulfide reduction act synergistically to facilitate lysosomal degradation of proteins. First, thiols activate the thiol-dependent lysosomal enzymes (e.g., cathepsins B, H, and L); and, second, thiols reduce protein disulfide linkages allowing proteins to unfold, thereby providing proteinases access to susceptible linkages within the substrate. Subsequently, Mego (25) demonstrated that cysteine accelerated the rate of intralysosomal proteolysis of the disulfide-rich protein BSA in intact mouse kidney lysosomes. Cysteine did not increase the rate of albumin breakdown, however, when the disulfide bonds of albumin had been previously reduced and alkylated, providing further evidence that thiols function to facilitate proteolysis of disulfide-rich proteins by reducing their disulfide linkages. In addition, Shen et al. (39) have found that the cytotoxicity of a conjugate in which methotrexate is linked to poly(D-lysine) through a disulfide spacer is dependent upon reduction of the disulfide linkage and that this reductive step likely occurs in either a prelysosomal or lysosomal compartment. In a review of the above studies concerning disulfide reduction in lysosomes, Lloyd (23) postulated that cysteine from the cytoplasm may be the physiological agent responsible for the intralysosomal reduction of protein disulfides.

Our present investigation demonstrates that a lysosomal transport system, highly specific for cysteine, provides a major route for the delivery of cysteine to the lysosomal compartment. The ability of this route to sequester large amounts of cysteine into lysosomes is evident from the cystine uptake studies demonstrating that a minimum of 50–60% of the total [^{35}S]cystine radioactivity taken up into cystinotic or normal fibroblasts is sequestered as cysteine into lysosomes (Table I), which comprise only 4% of the intracellular volume of normal human fibroblasts and 10% of the intracellular volume of cystinotic fibroblasts (11). This large degree of lysosomal cysteine sequestration occurs rapidly, being observed within the first 2 min of incubating intact fibroblasts with [^{35}S]cystine. Further evidence of the ability of this transport system to deliver cysteine to the lysosomal compartment is indicated by the ^{35}S chase experiments demonstrating that the addition of unlabeled cystine to culture medium produces an accelerated loss of intracellular [^{35}S]cystine from cystinotic fibroblasts whereas homocystine is without effect (Fig. 8 *B*).

The interchange between cystine and cysteine within the fibroblast appears to be a cyclic process, designed to address the requirements for cellular metabolism within both the cytosolic and lysosomal compartments (see Fig. 9). Cystine is transported across the plasma membrane into the cytosol by the X^-_{CG} transport system (3–5). Upon entering the cytosol, cystine is reduced by GSH to form cysteine and the mixed disulfide of glutathione and cysteine. Although low levels of this latter product have been observed in fibroblasts (3, 37), the majority of the mixed disulfide of glutathione and cysteine appears to be metabolized to other cellular constitu-

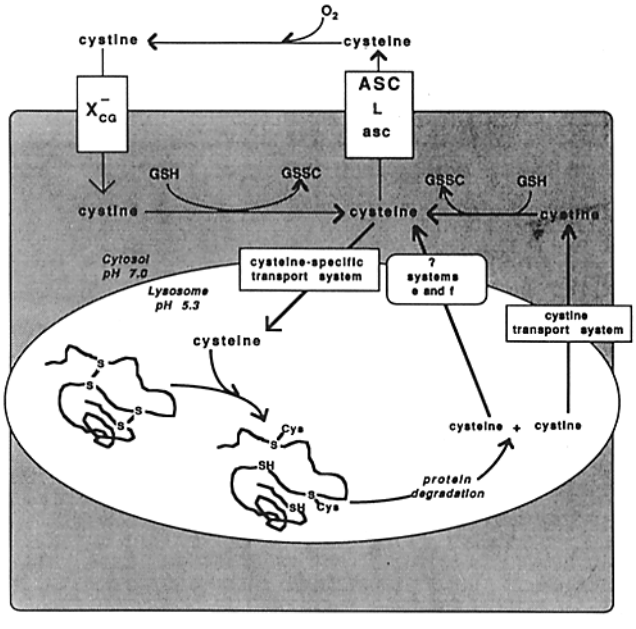

Figure 9. Schematic diagram showing (*a*) the interconversions between cystine and cysteine in different cellular compartments, (*b*) the role of transport systems in maintaining this cycle, and (*c*) the role of cysteine in supporting lysosomal proteolysis. Shaded area represents the cytosol whereas the clear area represents the lysosomal compartment; *ASC*, *L*, *asc*, and X^-_{CG} are plasma membrane amino acid transport systems.

ents, possibly by enzymes similar to those characterized in rat liver and bovine kidney extracts (7, 12, 46). Cysteine formed in the cytosol can be transported back into the extracellular space where it is reoxidized to form cystine (2). Cytosolic cysteine can also be used for the synthesis of gluthathione and proteins, metabolized for the production of other cellular constituents, or, as we have now shown, sequestered into the lysosomal compartment by the cysteine-specific lysosomal transport system to aid lysosomal proteolysis. Once within the lysosomal compartment, cysteine can react with protein disulfide bridges, as suggested by Lloyd (23), leading first to the formation of equimolar amounts of protein-linked cysteine and cystine at the site of each original disulfide bridge, and, after proteolysis, released into the lysosome as free cystine and cysteine. This cystine formed in the lysosome is then transported out of the lysosome into the cytosol by the lysosomal cystine transport system known to be defective in nephropathic cystinosis. Upon reaching the cytosol, cystine is reduced back to cysteine by GSH. No net production of intralysosomal cystine from cysteine occurs via this mechanism. This model accounts for the observation of Thoene and co-workers (42, 43, 45) that cystinotic fibroblasts accumulate lysosomal cystine from the degradation of disulfide-rich proteins but not from degrading cysteine-rich proteins.

We now see that the lysosomal membrane contains two highly specific transport systems involved in regulating the flow of cystine and cysteine between the intralysosomal space and the cytosol. Especially striking is how these two transport systems have adapted their properties to use the differences in pH and reducing environment, which exist between the cytosolic and lysosomal compartments, to direct their flow of substrate. (*a*) The pH dependence of the cysteine-specific lysosomal transport system appears to be used in conjunction with the difference in pH between the cytosol and intralysosomal space so as to direct net cysteine flow into lysosomes from the cytosol. We have not elucidated the basis of this pH dependence but our initial results with protonophores suggest that lysosomal cysteine transport may be coupled to the movement of protons or other ions across the lysosomal membrane. (*b*) At the neutral pH of the cytosol and extracellular medium, two ionic forms of cystine predominate: the zwitterionic, tetrapolar form and the anionic, tripolar form. It is this latter, anionic form of cystine, which is recognized by the plasma membrane X^-_{CG} transport system (4, 5). In contrast, within the acidic milieu of the lysosomal interior only the zwitterionic, tetrapolar form of cystine predominates and accordingly the specificity of the lysosomal cystine transport system appears to be directed towards this tetrapolar form of cystine (15), thus allowing it to function in accordance with the restrictions imposed by the acidic environment of the intralysosomal space. The net flow of lysosomal cystine transport is essentially unidirectional outward as a consequence of the reducing environment of the cytosol and the lysosome/cytosol pH gradient.

The manner by which cysteine is able to egress from lysosomes remains to be determined. Lysosomal cysteine exodus could be facilitated by lysosomal systems e and f, which both recognize a broad range of small neutral amino acids. For the present time, we have shown these two transport systems as being likely candidates for mediating lysosomal cysteine exodus in Fig. 9. The lysosomal cysteine-specific transport system is the first transport system to be described which appears to function for the net delivery of its substrate into the lysosomal compartment. Rome and co-workers (1, 34) however, have characterized a lysosomal membrane enzyme, acetyl-CoA (α-glucosaminide *N*-acetyltransferase), which catalyzes the transfer of the acetyl group of cytosolic acetyl-CoA to terminal α-linked glucosamine residues of heparan sulfate within the lysosome.

In 1976, Thoene et al. (44) demonstrated depletion of cystine from nephropathic cystinotic cells by treatment with cysteamine which reacts with intralysosomal cystine to form cysteine and the mixed disulfide of cysteamine and cysteine. Subsequently, we showed that the mixed disulfide of cysteamine and cysteine is recognized by lysosomal system c which serves for removal of this mixed disulfide from the lysosome (29, 31). The manner by which cysteamine is able to enter lysosomes, however, has not been determined. Our analog inhibition analysis (Table II) indicates that cysteamine is recognized by the lysosomal cysteine transport system by virtue of its ability to strongly inhibit [^{35}S]cysteine uptake, but whether cysteamine is actually transported by this route remains to be determined. Thus, these studies provide a focus for future investigations to determine if the lysosomal cysteine-specific transport system plays an important role in the delivery of cysteamine to the lysosomal compartment and allow for a more complete understanding of the basis of cysteamine therapy in the treatment of cystinosis.

This work was supported by National Institutes of Health grant DK25548.

Received for publication 22 May 1989 and in revised form 20 September 1989.

References

1. Bame, K. J., and L. H. Rome. 1986. Acetyl-coenzyme A:α-glucosaminide N-acetyltransferase: evidence for an active site histidine residue. *J. Biol. Chem.* 261:10127-10132.
2. Bannai, S., and T. Ishii. 1988. A novel function of glutamine in cell culture: utilization of glutamine for the uptake of cystine in human fibroblasts. *J. Cell. Physiol.* 137:360-366.
3. Bannai, S., and E. Kitamura. 1980. Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. *J. Biol. Chem.* 255:2372-2376.
4. Bannai, S., and E. Kitamura. 1981. Role of proton dissociation in the transport of cystine and glutamate in human diploid fibroblasts in culture. *J. Biol. Chem.* 256:5770-5772.
5. Bannai, S., and N. Tateishi. 1986. Role of membrane transport in metabolism and function of glutathione in mammals. *J. Membr. Biol.* 89:1-8.
6. Bernar, J., F. Tietze, L. D. Kohn, I. Bernardini, G. S. Harper, E. F. Grollman, and W. A. Gahl. 1986. Characteristics of a lysosomal membrane transport system for tyrosine and other neutral amino acids in rat thyroid cells. *J. Biol. Chem.* 261:17107-17112.
7. Chang, S. H., and D. R. Wilken. 1966. Participation of the unsymmetrical disulfide of coenzyme A and glutathione in an enzymatic sulfhydryl-disulfide interchange: particle purification and properties of the bovine kidney enzyme. *J. Biol. Chem.* 241:4251-4260.
8. Christensen, H. N. 1975. *In* Biological Transport. 2nd edition. W. A. Benjamin, Inc., Reading, MA. 456-460.
9. Cleland, W. W. 1979. Statistical analysis of enzyme kinetic data. *Methods Enzymol.* 63:103-139.
10. Edmondson, J. W., L. Lumeng, and T. K. Li. 1977. Direct measurement of active transport systems for alanine in freshly isolated rat liver cells. *Biochem. Biophys. Res. Commun.* 76:751-757.
11. Elferink, R. P., E. Harms, A. Strijland, and J. M. Tager. 1983. The intralysosomal pH in cultured human skin fibroblasts in relation to cystine accumulation in patients with cystinosis. *Biochem. Biophys. Res. Commun.* 116:154-161.
12. Eriksson, S. A., and B. Mannervik. 1970. The reduction of the L-cysteine-glutathione mixed disulfide in rat liver: involvement of an enzyme catalyzing thiol-disulfide interchange. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 7:26-28.
13. Gahl, W. A., N. Bashan, F. Tietze, I. Bernardini, and J. D. Schulman. 1982. Cystine transport is defective in isolated leukocyte lysosomes from patients with cystinosis. *Science (Wash. DC).* 217:1263-1265.
14. Gahl, W. A., F. Tietze, N. Bashan, R. Steinherz, and J. D. Schulman.

1982. Defective cystine exodus from isolated lysosome-rich fractions of cystinotic leukocytes. *J. Biol. Chem.* 257:9570–9575.
15. Gahl, W. A., F. Tietze, N. Bashan, I. Bernardini, D. Raiford, and J. D. Schulman. 1983. Characteristics of cystine counter-transport in normal and cystinotic lysosome-rich leucocyte granular fractions. *Biochem. J.* 216:393–400.
16. Gahl, W. A., M. Renlund, and J. G. Thoene. 1989. Lysosomal transport disorders: cystinosis and sialic acid storage disorders. *In* The Metabolic Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Inc., New York. 2619–2641.
17. Griffiths, P. A., and J. B. Lloyd. 1979. Evidence for lysosomal reduction of cystine residues. *Biochem. Biophys. Res. Commun.* 89:428–434.
18. Harms, E., H. Kern, and J. A. Schneider. 1980. Human lysosomes can be purified from diploid skin fibroblasts by free-flow electrophoresis. *Proc. Natl. Acad. Sci. USA.* 77:6139–6143.
19. Jonas, A. J., A. A. Greene, M. L. Smith, and J. A. Schneider. 1982. Cystine accumulation and loss in normal, heterozygous, and cystinotic fibroblasts. *Proc. Natl. Acad. Sci. USA.* 79:4442–4445.
20. Jonas, A. J., R. J. Speller, P. B. Conrad, and W. P. Dubinsky. 1989. Transport of *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine by rat liver lysosomes. *J. Biol. Chem.* 264:4953–4956.
21. Kooistra, T., P. C. Millard, and J. B. Lloyd. 1982. Role of thiols in degradation of proteins by cathepsins. *Biochem. J.* 204:471–477.
22. Lloyd, J. B. 1970. Studies on the permeability of rat liver lysosomes to carbohydrates. *Biochem. J.* 115:703–707.
23. Lloyd, J. B. 1986. Disulphide reduction in lysosomes. *Biochem. J.* 237:271–272.
24. Mancini, G. M. S., H. R. de Jonge, H. Galjaard, and F. W. Verheijen. 1989. Characterization of a proton driven carrier for sialic acid in the lysosomal membrane: evidence for a group specific transport system for acidic monosaccharides. *J. Biol. Chem.* 264:15247–15249.
25. Mego, J. L. 1984. Role of thiols, pH and cathepsin D in the lysosomal catabolism of serum albumin. *Biochem. J.* 218:775–783.
26. Nyberg, E., and J. T. Dingle. 1970. Endocytosis of sucrose and other sugars by cells in culture. *Exp. Cell. Res.* 63:43–52.
27. Oshima, R. G., W. J. Rhead, J. G. Thoene, and J. A. Schneider. 1975. Cystine metabolism in human fibroblasts: comparison of normal, cystinotic, and γ-glutamylcysteine synthetase-deficient cells. *J. Biol. Chem.* 251:4287–4293.
28. Pisoni, R. L., and J. G. Thoene. 1989. Detection and characterization of a nucleoside transport system in human fibroblast lysosomes. *J. Biol. Chem.* 264:4850–4856.
29. Pisoni, R. L., J. G. Thoene, and H. N. Christensen. 1985. Detection and characterization of carrier-mediated cationic amino acid transport in lysosomes of normal and cystinotic human fibroblasts: role in therapeutic cystine removal? *J. Biol. Chem.* 260:4791–4798.
30. Pisoni, R. L., K. S. Flickinger, J. G. Thoene, and H. N. Christensen. 1987. Characterization of carrier-mediated transport systems for small neutral amino acids in human fibroblast lysosomes. *J. Biol. Chem.* 262:6010–6017.
31. Pisoni, R. L., J. G. Thoene, R. M. Lemons, and H. N. Christensen. 1987. Important differences in cationic amino acid transport by lysosomal system c and system y^+ of the human fibroblast. *J. Biol. Chem.* 262:15011–15018.
32. Renlund, M., F. Tietze, and W. A. Gahl. 1986. Defective sialic acid egress from isolated fibroblast lysosomes of patients with Salla Disease. *Science (Wash. DC).* 232:759–762.
33. Rome, L. H., A. J. Garvin, M. M. Allietta, and E. F. Neufeld. 1979. Two species of lysosomal organelles in cultured human fibroblasts. *Cell.* 17:143–153.
34. Rome, L. H., D. F. Hill, K. J. Bame, and L. R. Crain. 1983. Utilization of exogeneously added acetyl coenzyme A by intact isolated lysosomes. *J. Biol. Chem.* 258:3006–3011.
35. Rosenblatt, D. S., A. Hosack, N. V. Matiaszuk, B. A. Cooper, and R. LaFramboise. 1985. Defect in vitamin B_{12} metabolism. *Science (Wash. DC).* 228:1319–1321.
36. Schneider, J. A., F.M. Rosenbloom, K. H. Bradley, and J. E. Seegmiller. 1967. Increased free-cystine content of fibroblasts cultured from patients with cystinosis. *Biochem. Biophys. Res. Commun.* 29:527–531.
37. Schulman, J. D., and K. H. Bradley. 1973. *In vitro* studies on cystinosis. *In* Cystinosis. J. D. Schulman, editor. DHEW publication no. 72-249. National Institutes of Health, Bethesda, MD. 131–132.
38. Schulman, J. D., K. H. Bradley, and J. F. Seegmiller. 1969. Cystine: compartmentalization within lysosomes in cystinotic leukocytes. *Science (Wash. DC).* 166:1152–1154.
39. Shen, W. C., H. J.-P. Ryser, and L. LaManna. 1985. Disulfide spacer between methotrexate and poly (D-lysine): a probe for exploring the reductive process in endocytosis. *J. Biol. Chem.* 260:10905–10908.
40. Smith, M. L., A. A. Greene, R. Potashnik, S. A. Mendoza, and J. A. Schneider. 1987. Lysosomal cystine transport: effect of intralysosomal pH and membrane potential. *J. Biol. Chem.* 262:1244–1253.
41. Smith, M. L., A. A. Greene, J. A. Schneider, R. L. Pisoni, and H. N. Christensen. 1989. Cystine exodus from lysosomes (cystinosis). *Methods Enzymol.* In press.
42. Thoene, J. G., and R. N. Lemons. 1980. Modulation of the intracellular cystine content of cystinotic fibroblasts by extracellular albumin. *Pediatr. Res.* 14:785–787.
43. Thoene, J. G., and R. M. Lemons. 1982. Cystine accumulation in cystinotic fibroblasts from free and protein-linked cystine but not cysteine. *Biochem. J.* 208:823–830.
44. Thoene, J. G., R. G. Oshima, J. C. Crawhall, D. L. Olson, and J. A. Schneider. 1976. Cystinosis: intracellular cystine depletion by aminothiols in vitro and in vivo. *J. Clin. Invest.* 48:180–189.
45. Thoene, J. G., R. G. Oshima, D. G. Ritchie, and J. A. Schneider. 1977. Cystinotic fibroblasts accumulate cystine from intracellular protein degradation. *Proc. Natl. Acad. Sci. USA.* 74:4505–4507.
46. Tietze, F. 1973. Enzymatic reduction of cystine and other disulfides. *In* Cystinosis. J. D. Schulman, editor. DHEW publication No. 72-249. National Institutes of Health, Bethesda, MD. 147–197.
47. Tietze, F., L. D. Kohn, A. D. Kohn, I. Bernardini, H. C. Andersson, M. D. Adamson, G. S. Harper, and W. A. Gahl. 1989. Carrier-mediated transport of monoiodotyrosine out of thyroid cell lysosomes. *J. Biol. Chem.* 264:4762–4765.