Important Differences in Cationic Amino Acid Transport by Lysosomal System c and System y+ of the Human Fibroblast*

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Superficial similarities led us to extend our designation for the transport of the plasma membrane for cationic amino acids, y+, to the lysosomal system also serving for such amino acids. Further study on the purified lysosomes of human skin fibroblasts leads us now to redesignate the lysosomal system as c (for cationic), rather than y+, to emphasize important contrasts. Lysosomal uptake of arginine at pH 7.0 was linear during the first 2 min, but attained a steady state in 6 min. This arginine uptake was Na+-independent and was tripled in rate when the lysosomes had first been loaded with the cationic amino acid analog S-2-aminoethyl-L-cysteine. Uptake was allowed to one-third when 2 mM MgATP was added to the incubation mixture. The following differences in cationic amino acid influx between lysosomal System c and the plasma membrane System y* became apparent: 1) arginine influx is increased 10-fold by raising the external pH from 5.0 to 7.0. This effect favors net entry of cationic amino acids under the H+ gradient prevailing in vivo. In contrast, arginine uptake across the plasma membrane is insensitive to pH changes in this range. 2) The $K_m$ of arginine uptake by lysosomal System c, 0.32 mM, is eight times that for System y* arginine uptake by the fibroblast. 3) Certain neutral amino acids in the presence of Na+ are accepted as surrogate substrates by System y*, but not by lysosomal system c. 4) Cationic amino acids in which the α-amino group is monomethylated or the distal amino group is quaternary, also D-arginine, are recognized by lysosomal System c, whereas System y* has little affinity for these analogs. This broader substrate specificity of lysosomal system c leads us to discover that thiocaine serves to deplete accumulated cysteine from cystinotic fibroblasts as effectively as does the therapeutic agent, cysteamine. The quaternary nitrogen of thiocaine renders the mixed disulfide formed when it reacts with cysteine unsatisfactory as a substrate for System y*.

Lysosomes are a major site for the intracellular degradation of many kinds of macromolecules. Several different pathways have been described which provide a continual flow to the lysosome of macromolecules derived from both intracellular and extracellular constituents (1). Escape of metabolites, formed within the lysosome as a consequence of macromolecular degradation, is necessary to avoid their accumulation. Studies during the last 5 years indicate that carrier-mediated transport plays a major role in the passage of small organic ways from across the lysosomal membrane. The majority of these studies have focused on transport of amino acids across the lysosomal membrane and have resulted in the characterization of transport systems for cysteine (2-5), for the small neutral amino acids (6) and for tyrosine and other bulky neutral amino acids (7). In addition, we have demonstrated the existence of a transport system mediating the passage of cationic amino acids across the lysosomes of human fibroblasts (8). This system, now provisionally named lysosomal system c, provides a route by which the therapeutic agent, cysteamine, decreases lysosomal cysteine accumulation in the genetic disease, nephropathic cystinosis, by allowing its escape in the form of the mixed disulfide of cysteine and cysteamine, which is recognized by this transport system. In the present investigation, analog inhibition experiments with purified human fibroblast lysosomes were designed to study lysosomal system c in greater detail. Arginine was selected as a model substrate because of its proved lack of reactivity with other noncationic amino acid transport systems (9, 10). Our results indicate that lysosomal system c differs in several important ways from system y* which serves for transport of cationic amino acids across the plasma membrane in many eukaryotes (11). We have taken advantage of the broader substrate specificity of lysosomal system c to find another aminothiol, thiocaine, which prove to be as effective as cysteamine in depleting cystinotic fibroblasts of their accumulated cysteine.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Percoll-purified Lysosomes—Normal (GM0010) and cystinotic (GM0090A) human fetal skin fibroblast cell lines were obtained from the Human Genetic and Mutant Cell Repository. Fibroblasts were grown and maintained in an atmosphere of 95% air/5% CO2 in 100-mm tissue culture dishes or 850-cm2 roller bottles in Coon's modification of Ham's F-12 medium (Hazelton Research Products, Inc.) supplemented with 10% fetal bovine serum. Lysosomes were purified on density gradients of Percoll as described previously (6) and generally resuspended in 100 mM citrate/Tris pH 7.0 buffer.

Arginine Uptake Experiments—In a typical time course, a 75-μl aliquot of [14C]L-arginine (342 mCi/mmol, 0.135 mM) in 0.25 M 1

Whenever a gene product is identified that corresponds to one of the amino acid transport systems heretofore designated by a single letter, we suggest, in order to provide a designation conventional for a gene product, that the letters AT (for amino acid transporter) be added to that single letter. Thus, a gene product corresponding to lysosomal System e be designated EAT. Where a transport system is already designated by three letters (e.g. ASC or asc) these three, capitalized, could serve for the gene product, adding another symbol as needed for identification, e.g. ASC II.

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sucrose was mixed with 150 μl of 50 mM citrate/Tris pH 7.0 buffer containing 0.125 mM sucrose and prewarmed at 37 °C. Time courses were initiated by the addition of 75 μl of ice-cold fibroblast lysosomes in 100 mM citrate/Tris pH 7.0 buffer. At the indicated time points, 18-μl aliquots were removed from the incubation mixture, added to 12 ml of ice-cold phosphate-buffered saline, and filtered through a GF/A glass fiber filter; the filter was washed twice with 12-ml portions of ice-cold phosphate-buffered saline and then counted for radioactivity in 10 ml of 3a70b scintillation fluid (Research Products International). Blanks were run for each experiment by substituting 100 mM citrate/Tris pH 7.0 buffer for the lysosomes in the incubation mixture. Aliquots removed from the blank incubations were filtered and washed in the same manner as for samples containing lysosomes. Radioactivity retained on the GF/A filters for these blank mixtures was subtracted from radioactivity retained on filters for lysosome-containing samples. Latent β-hexosaminidase activity was measured for each lysosomal preparation as described previously (8). Uptake is expressed as picomoles of arginine taken up during the indicated time period per unit of latent β-hexosaminidase activity. Analogue inhibition experiments were carried out as described previously (6), except that uptakes were for 1.5 min.

Analysis of Kinetic Data—The non saturable component of uptake was determined by secondary analysis of initial velocity kinetic data using a Fortran program (12) applying the Gauss-Newton nonlinear least squares method as described in the following equation.

\[
\log v = \log \left( \frac{V_{\max} - S}{K_s + S} + K_n S \right)
\]

**Reaction of [3H]-Cystine with Aminothiols**—The granular fraction from cystinotic fibroblasts was prepared as described above and incubated with 0.067 mM [3H]-cystine dimethyl ester (840 mCi/mmol) at 37 °C for 60 min in a volume of 0.35 ml in 10 mM Mops/Tris pH 7.0 buffer containing 0.275 M mannitol (wash buffer). Portions were incubated for 15 min at 22 °C with an equal volume of wash buffer. Incubations were terminated by dilution to 1.5 ml with 10 mM sodium phosphate pH 7.0 buffer. The granular suspension was then diluted to 1.5 ml with ice-cold 20 mM Mops/Tris pH 7.0 buffer containing 0.25 mM sucrose and 1 mM Na₂EDTA. The granular suspension was then diluted to 1.5 ml with ice-cold 20 mM Mops/Tris pH 7.0 buffer containing 0.257 mM mannitol (wash buffer) and centrifuged at 15,600 × g for 10 min. The supernatant was discarded and this step repeated. The final [3H]-cystine-loaded granular pellet was suspended in 0.25 ml of ice-cold wash buffer and then 0.078-ml portions were incubated for 15 min at 22 °C with an equal volume of wash buffer or either 2 mM cysteamine or 2 mM thiocholine iodide in wash buffer. Incubations were terminated by dilution to 1.5 ml with ice-cold wash buffer containing 10 mM NEM and centrifuged for 12 min at 4 °C and 15,600 × g. Supernatants were discarded and the pellets resuspended in 0.1 ml of 10 mM sodium phosphate pH 7.0 buffer containing 10 mM NEM. Lysosomes were lysed by the addition of 10 μl of 1% Triton with vortexing, and 11 μl of 40% sulfosalicylic acid were added at 20 °C and stored at −20 °C until analysis. The supernatants were spotted on paper along with 30 nmol each of the standards and cysteine-NEM, the mixed disulfide of cysteamine and cysteine, and the mixed disulfide of thiocholine and cysteine. The paper was visualized by spraying the paper with ninhydrin. Each lane was run and dried in a vacuum oven. The position of internal standards was visualized by spraying the paper with ninhydrin. Each lane was then cut into 1-cm segments which were counted in 10 ml of scintillation fluid to determine the distribution of radioactivity.

**Synthesis of Mixed Disulfides**—The mixed disulfide of cysteamine and cystine was synthesized and purified as described previously (8). The mixed disulfide of thiocholine and cysteine was synthesized and purified in exactly the same way except that thiocholine iodide was substituted for cysteamine on an equivalent molar basis. The mixed disulfide of thiocholine and cysteine, so obtained, migrated as a single spot on high voltage electrophoresis in 6% formic acid with an Rf indistinguishable from that of the mixed disulfide of cysteamine and cystine.

**Synthesis of Methyl Esters**—[3H]-Cystine dimethyl ester and 2-aminomethyl-l-cystine monomethyl ester were synthesized according to Steinherz et al. (13) and were stored at −20 °C in absolute methanol. Immediately before experimental use, aliquots from the methyl ester solutions were evaporated to dryness under a stream of N₂ gas and redissolved in the appropriate buffer.

**Miscellaneous**—Osmolarity measurements were performed with an Advanced Instruments freezing point depression osmometer. [3H]-Arginine (342 mCi/mmol) and [3H]-cystine (830 mCi/mmol) were purchased from Amersham Corp.; [3H]-lysine was from Du Pont New England Nuclear. Percoll was obtained from Pharmacia (Biotechnology, Inc., Piscataway, N.J.). Thiocholine iodide and d-arginine were from ICN, ethylthimethyl-l-lysine was purchased from Behring Diagnostics and α-N-methyl-l-arginine from Vega. DL-α-Hydroxy-α-aminocaproate was obtained from Cyclo Chemical Co. The cysteamine, nucleotides, and other amino acids were purchased from Sigma.

**RESULTS**

Time courses of arginine uptake into fibroblast lysosomes at pH 7.0 are shown in Fig. 1. Arginine uptake was found to be a Na⁺-independent process displaying virtually identical time courses whether performed in buffer containing 0.125 mM sucrose or in buffer in which sucrose was replaced by 77 mM NaCl. Under these conditions, lysosomal arginine uptake proceeded linearly during the first 2 min of observation and attained a steady state by 5 min. In contrast, arginine influx was retarded considerably when 2 mM MgATP was present in the incubation mixture. This effect of MgATP appears rapidly, being noticeable within the first minute of the time course. MgATP was found to retard also the lysosomal uptake of lysine 3-fold (data not shown). In contrast, 2 mM external MgATP doubled the efflux of lysine from human fibroblast lysosomes (8). Thus the changes which occur on incubation of fibroblast lysosomes with MgATP result in accelerating the efflux and retarding the influx of amino acids transported by lysosomal system c.

In Fig. 2, the specificity for MgATP in retarding arginine uptake is seen in that neither MgADP nor the nonhydrolyzable ATP analog, β,γ-imidoadenosine-5′-triphosphosphate, has a measurable effect on arginine uptake. The result with this latter analog indicates that cleavage between the β- and γ-phosphate groups of ATP is necessary in order for the effect of ATP to be demonstrated. Although we have no information on the actual mechanism by which MgATP has its effect on lysosomal system c, a similar nucleotide specificity has been observed by Ohkuma et al. (14) for acidification by the proton pump of rat liver lysosomes. N-Ethylmaleimide, which has
arginine, remains virtually unchanged over this pH range; hence this response to pH could be due either to titration of important groups on the transport protein itself or to pH-dependent changes in the lysosomal environment which could affect transport (e.g. membrane potential, ion, or pH gradients). The intralysosomal pH in the human fibroblast has been measured as 5.3 (15, 16), whereas the cytoplasmic pH is near 7.0 (17). As a consequence, the pH activity curve of lysosomal system c favors net entry of cationic amino acids into the lysosome because the normal H+ distribution is 10 times more favorable for the inward than for the outward flux.

We had shown previously that a trans-stimulation property is associated with lysine exodus from fibroblast lysosomes and is one of the distinguishing characteristics of lysosomal system c in the human fibroblast (8). In order to test whether arginine uptake is subject also to trans-stimulation, arginine uptake into unloaded control lysosomes was compared with uptake into lysosomes loaded with S-2-aminoethyl-L-cysteine using the methyl ester derivative to facilitate loading of lysosomes to high concentrations (18). The results, displayed in Fig. 4, indicate that [14C]-arginine uptake into lysosomes preloaded with 2-aminoethyl-L-cysteine was nearly 3-fold greater than uptake into nonloaded control lysosomes. Furthermore, trans-stimulated arginine uptake was subject to retardation by the action of MgATP. This evidence strongly suggests that arginine uptake into Percoll-purified lysosomes is mediated by lysosomal system c and that the trans-stimulation property can be observed for either direction of transport. Additional evidence that transport was directed into intact vesicular structures was derived from experiments in which arginine uptake was found to vary inversely with the osmolarity of the incubation medium (data not shown).

been found to inhibit this pump (14), could not be studied for its ability to abrogate the effect of MgATP on arginine uptake because N-ethylmaleimide by itself inhibits arginine uptake. The initial rate of arginine uptake into fibroblast lysosomes was found to be extremely sensitive to the pH of the extralysosomal medium (Fig. 3). Uptake was 10-fold greater at pH 7.0 than at 5.0 and displayed a broad maximum over the range from pH 7.0 to 8.0. The state of ionization of the substrate,

arghinine, remains virtually unchanged over this pH range; hence this response to pH could be due either to titration of important groups on the transport protein itself or to pH-dependent changes in the lysosomal environment which could affect transport (e.g. membrane potential, ion, or pH gradients). The intralysosomal pH in the human fibroblast has been measured as 5.3 (15, 16), whereas the cytoplasmic pH is near 7.0 (17). As a consequence, the pH activity curve of lysosomal system c favors net entry of cationic amino acids into the lysosome because the normal H+ distribution is 10 times more favorable for the inward than for the outward flux.

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A Michaelis-Menten plot (Fig. 5A) of the initial rate of lysosomal arginine uptake as a function of substrate concentration demonstrates that the uptake process is saturable and yields a linear Lineweaver-Burk plot (Fig. 5B). The apparent kinetic constants for lysosomal arginine uptake, $K_m = 0.32 \text{ mM} \pm 0.03$ and $V_{max} = 3.2 \text{ pmol of arginine/min/hexosaminidase}$, were obtained by fitting the estimated initial uptake velocities to Equation 1 (see "Experimental Procedures"). This $K_m$ for arginine uptake by lysosomal system c is approximately 8-fold larger than that observed for arginine uptake into human fibroblasts by system $y^+$ (9). As observed with lysosomal uptake of several other amino acids (6), the $V_{max}$ tends to decline with age of cells in culture and hence is not a fixed kinetic parameter.

Analogue inhibition experiments were performed to assess the substrate specificity of lysosomal system c using arginine as a model substrate. The results, shown in Table I, demonstrate that arginine uptake is strongly inhibited by various cationic amino acids, whereas neutral and anionic amino acids had very little effect. The total lack of inhibitory effect of L-lysine methyl ester points to a requirement for the $\alpha$-carboxyl group of the cationic amino acid to be in a free state for recognition at the transport receptor site. Cationic amino acids with side chains varying in length between those of ornithine and homoarginine demonstrate nearly the same ability to inhibit arginine uptake, whereas 2,4-diaminobutyrate produces a much weaker inhibition. This suggests that recognition of cationic amino acids by lysosomal system c requires side chains with a minimum length of five carbons. The complete lack of inhibition by 1,2,3-diaminopropionate is likely due to the combination of the short side chain length coupled with the low $pK'$ of its distal amino group (20) such that at pH 7.0 only about half of the diaminopropionate is present in the cationic form. A distinguishing feature of arginine uptake by lysosomal system c is its inhibition by D-arginine, $\alpha-N$-methyl-L-arginine and $\epsilon$-trimethyl-L-lysine. These analogs at 3.33 mM gave 50–75% inhibition of 0.03 mM [14C]-arginine uptake. This contrasts sharply with arginine uptake by system $y^+$ of the plasma membrane which is not inhibited to any significant degree by $\alpha-N$-methyl-L-arginine or $\epsilon$-trimethyl-L-lysine and recognizes D-arginine approximately one-twentieth as well as L-arginine (9).

Certain neutral amino acids in the presence of alkali metal ions have been shown to inhibit uptake of cationic amino acids by system $y^+$ in the Ehrlich ascites tumor cell, the rabbit reticulocyte, Saccharomyces cerevisiae (20–24), and the human skin fibroblast (9). Recognition of these neutral amino acids is thought to be facilitated by the binding of the alkali metal ion to the site on the transport protein receptor site which would normally be occupied by the cationic group of the basic amino acid side chain. To determine if this property is associated also with lysosomal system c, we tested the ability of homoserine or $\epsilon$-hydroxy-$\alpha$-aminocapric acid to inhibit lysosomal arginine uptake in the presence of absence of 77 mM NaCl. The results, shown in Table II, demonstrate that neither of these neutral amino acids inhibit lysosomal arginine uptake, and this lack of inhibitory effect is not changed by adding sodium chloride.

Analogs from Table I, which substantially inhibited [14C]-arginine uptake, were shown to act as competitive inhibitors by the following analysis. For all such analogs tested, five different concentrations of inhibitor were chosen for their ability to inhibit either 0.02, 0.05, 0.10, or 0.25 mM [14C]-arginine uptake. Results, when displayed in a double-reciprocal plot, yielded inhibition lines intersecting on the $y$-axis. Fig. 6A shows such a plot for the inhibition of [14C]-arginine uptake by several different concentrations of lysine. The slopes of individual inhibition curves from the double-reciprocal analyses were then plotted as a function of the inhibitor.
The uptake of 0.03 mM $[^{14}C]L$-arginine into fibroblast lysosomes was measured in the presence or absence of each of the listed amino acids (3.33 mM) during a 1.5-min incubation period at 37 °C. Uptakes were performed in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 M sucrose. The listed amino acids were present at a concentration of 3.33 mM except for NaCl. The uptake of 0.04 mM $[^{14}C]L$-arginine into fibroblast lysosomes was measured in the presence or absence of NaCl.

### Table I

| Amino Acid       | % of uninhibited rate |
|------------------|-----------------------|
| L-Arginine       | 11 ± 1                |
| D-Arginine       | 46 ± 5                |
| L-Ornithine      | 23 ± 1                |
| L-Homoarginine   | 22 ± 2                |
| $\alpha$-N-Methyl-L-arginine | 27 ± 2     |
| S-2-Aminoethyl-L-cysteine | 32 ± 1  |
| L-Lysine         | 30 ± 4                |
| $\epsilon$-Trimethyl-L-lysine | 60 ± 5   |
| L-Lysine methyl ester | 100 ± 2 |
| L-2,4-Diaminobutyrate | 62 ± 2   |
| L-2,3-Diaminopropionate | 106 ± 1  |
| L-Histidine      | 72 ± 4                |
| L-Methionine     | 82 ± 5                |
| L-Aspartate      | 87 ± 4                |
| L-Alanine        | 91 ± 3                |
| L-Proline        | 85 ± 1                |
| L-Leucine        | 91 ± 4                |

### Table II

| Amino Acid       | % of uninhibited rate |
|------------------|-----------------------|
| L-Arginine       | 11 ± 1                |
| L-Homoserine     | 100 ± 14              |
| D,L-\(\epsilon\)-Hydroxy-\(\alpha\)-aminocaproate | 94 ± 2                   |

**Fig. 6. Inhibition of lysosomal arginine uptake by lysine.** Fibroblast lysosomes were incubated with either 0, 0.3, 0.62, 1.25, or 2.50 mM L-lysine for 1.5 min at 37 °C with $[^{14}C]L$-arginine of the indicated concentration in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 M sucrose. At the completion of the incubation period, lysosomes were filtered and washed, and the amount of radioactive arginine taken up was measured. A, double-reciprocal plot of the initial rate of $[^{14}C]L$-arginine uptake as a function of the arginine concentration for the different concentrations of lysine which were used; B, slope replot, the slope of each curve from the above double-reciprocal plot is graphed as a function of the L-lysine concentration. The negative of the x-axis intercept yields a $K_i$ of 1.0 mM for L-lysine.

Concentration (Fig. 6) resulting in a linear proportionality indicative of a competitive-type inhibition. The $K_i$ values obtained from the slope replots for each of the different inhibitors of arginine uptake are listed in Table III. These inhibition constants reveal that monomethylation of the $\alpha$-amino group of arginine only slightly reduces the affinity of L-arginine for lysosomal system c. Recognition of D-arginine is about one-fourth that of L-arginine, indicating only a moderate discrimination by lysosomal system c with regard to stereoisomers. The affinity of L-lysine, L-ornithine, and 2-aminoethyl-L-cysteine for inhibiting lysosomal system c is one-third of that seen for L-arginine. Trimethylation of the $\epsilon$-amino group of lysine lowers to one-fourth its ability to inhibit $[^{14}C]L$-arginine uptake.

In the inherited genetic disease, nephropathic cystinosis, cystine accumulates in the lysosomes of affected persons due to a defect in lysosomal cystine transport (2-4). It has been demonstrated that the accumulated intralysosomal cystine could be depleted by treatment with cysteamine which was postulated to react with cystine to form cysteine and the mixed disulfide of cysteine and cysteine. We have recently shown that the mixed disulfide cysteamine and cysteine is recognized by lysosomal system c, which thus serves for removal of this mixed disulfide from the lysosome (8). Our present finding that lysosomal system c recognizes $\epsilon$-trimethyl-L-lysine led us to investigate whether the aminothiol, thiocholine, could also serve to deplete cystinotic lysosomes of their accumulated cystine. Cystinotic fibroblasts were labeled with $[^{35}S]L$-cysteine for 18 h, washed, and then incubated in cystine-free culture medium in the presence or absence of

| Amino Acid       | % of uninhibited rate |
|------------------|-----------------------|
| $[^{14}C]L$-arginine |                        |
| $[^{35}S]L$-cysteine |                        |
TABLE III

Competitive inhibition constants of selected amino acid analogs for inhibiting \(^{14}C\)L-arginine uptake into fibroblast lysosomes

Uptakes (1.5 min) of four different concentrations of \(^{[14}C\)L-arginine were measured in the presence or absence of five different concentrations of inhibitor as described and shown in Fig. 6A. The slopes of the individual inhibition curves from the Lineweaver-Burk plots were then graphed as a function of the corresponding inhibitor concentration and the negative of the x axis intercept was calculated to determine the \(K_i\) for each inhibitor. The results shown are the average of two determinations.

| Analog                  | \(K_i\) (mM) |
|-------------------------|-------------|
| L-Arginine              | 0.34 ± 0.08 |
| \(\alpha\)-Methyl-L-arginine | 0.60 ± 0.01 |
| L-Homoarginine          | 0.80 ± 0.04 |
| L-Ornithine             | 1.1 ± 0.2   |
| L-Lysine                | 1.1 ± 0.11  |
| 2-Aminoethyl-L-cysteine | 1.2 ± 0.04  |
| D-Arginine              | 1.5 ± 0.04  |
| \(\epsilon\)-Trimethyl-L-lysine | 4.5 ± 0.50  |

**FIG. 7. Effect of thiocholine and cysteamine on the loss of \(^{35}S\)L-cystine from human cystinotic fibroblasts.** Confluent monolayers of cystinotic fibroblasts in 60-mm dishes were incubated for 18 h at 37 °C in 3 ml of Coon’s modified F-12 medium containing 15% fetal calf serum and \(^{35}S\)L-cystine (1.25 \times 10^7 cpm/ml). The radioactive medium was then discarded, the monolayers were washed three times with phosphate-buffered saline and at time 0, incubated at 37 °C in cystine-free F-12 medium containing 15% dialyzed fetal calf serum and either 1 mM cysteamine, 1 mM thiocholine iodide, or 1 mM 2-mercaptopimidozole. The cells were harvested by trypsin treatment at the times indicated and immediately broken by sonic disruption in 100 mM sodium phosphate pH 7.4 buffer containing 5 mM NEM. Cellular proteins were precipitated by centrifugation after addition of sulfosalicylic acid to 3%, the supernatants were spotted on paper along with internal standards and subjected to high voltage electrophoresis as described under “Experimental Procedures.” Radioactive cystine spots were cut out and counted.

**DISCUSSION**

Our previous studies demonstrated a system mediating the transport of cationic amino acids across the lysosomal membrane of the human fibroblast (8). This system, which we now provisionally term lysosomal system c (c for cationic), was shown to be similar to system y- of the plasma membrane in that both systems mediate the transport of certain cationic amino acids and both display a trans-stimulation property. In the present investigation, analog inhibition studies of a model substrate, L-arginine, demonstrate that lysosomal system c bears important differences to system y- of the plasma membrane: (i) lysosomal system c has a broader substrate specificity than system y-, accepting cationic amino acids in which the \(\alpha\)-amino group is monomethylated or the \(\epsilon\)-amino group is trimethylated; these structures are not accepted by system y- (9); in addition, system y- is much more specific to the L-stereoisomer of arginine, whereas lysosomal system c recognizes L-arginine with only 4-fold greater affinity than D-arginine; (ii) arginine uptake by system y- of the human fibroblast is insensitive to changes in pH from pH 5 to 8 (11). In contrast, arginine uptake by lysosomal system c is highly sensitive to pH over this range, displaying 10-fold greater uptake at pH 7 than at pH 5; (iii) the \(K_m\) for arginine uptake by lysosomal system c is 8-fold greater than that found for

choline, this latter product being structurally analogous to \(\epsilon\)-trimethyl-L-lysine. To examine the intralysosomal reaction of thiocholine with cystine, lysosomes were loaded with \(^{[3}H\)L-cystine and then incubated in the presence or absence of either 1 mM cysteamine or 1 mM thiocholine iodide for 15 min at 22 °C. The acid-soluble portion of the reaction mixtures were then subjected to high voltage electrophoresis. The distribution of radioactivity present in \(^{[3}H\)L-cystine-loaded lysosomes not treated with either thiol is shown in Fig. 9, panel A. Nearly all the radioactivity co-migrates as a single peak with the cystine standard. In contrast, in cysteamine-treated lysosomes (panel B), or thiocholine-treated lysosomes (panel C), radioactivity co-migrating with the cystine standard is reduced to one-half to one-third of that seen in panel A. In addition, two new peaks are seen: a large peak co-migrating with the cysteine-NEM standard and a fast moving peak co-migrating with the internal standard of the respective mixed disulfide (MD). Hence, thiocholine reacts with cystine in a manner similar to that of cysteamine.

The degree of recognition of the mixed disulfide of cysteamine and cysteine and the mixed disulfide of thiocholine and cysteine by lysosomal system c was determined by testing various concentrations of the respective mixed disulfides for their ability to inhibit the uptakes of either 0.02, 0.05, 0.10, or 0.25 mM \(^{[14}C\)L-arginine by fibroblast lysosomes as described in the legend to Fig. 6. The different inhibitor curves from the double-reciprocal plot intersected on the y-axis, similar to Fig. 6A, and a replot of the slopes of individual inhibition curves as a function of the inhibitor concentration was linear from which plots the \(K_i\) values shown in Table IV were derived. The mixed disulfide of cysteamine and cysteine, which is structurally analogous to L-lysine and 2-aminoethyl-L-cysteine, demonstrated the same \(K_i\) as these two amino acids. In contrast, the mixed disulfide of thiocholine and cysteine, analogous to \(\epsilon\)-trimethyl-L-lysine in structure, proved to be considerably more effective as an inhibitor, displaying a \(K_i\) of 1.3 mM compared to 4.5 mM for \(\epsilon\)-trimethyl-L-lysine. In a separate experiment (data not shown), the mixed disulfide of thiocholine and cysteine at a concentration of 10 mM did not inhibit the uptake of 0.03 mM \(^{[14}C\)L-arginine into intact human fibroblasts by system y-.

As shown in Fig. 8, reaction of thiocholine with cysteine, if similar to that of cysteamine, would result in the formation of cystine and the mixed disulfide of cysteamine and thio-
Blasts were loaded with $[^3H]^{-\text{cystine}}$ and then incubated with either addition of sulfosalicylic acid to determine the distribution of radioactivity. MD, mixed disulfides.

These differences could arise if lysosomal system c and system y+ are products of different genes or, if encoded by the same gene, differ significantly in post-translational modifications. Alternatively, binding of a specific cellular component to one of the transport proteins could alter its substrate specificity and kinetic parameters analogous to that seen upon binding of α-lactalbumin to β-galactosyl transferase (28). Recently, arginine uptake by system y+ (9); and (iv) certain neutral amino acids in the presence of selected nonmonovalent cations are recognized by system y+ but not by lysosomal system c. These differences could arise if lysosomal system c and system y+ are products of different genes or, if encoded by the same gene, differ significantly in post-translational modifications. Alternatively, binding of a specific cellular component to one of the transport proteins could alter its substrate specificity and kinetic parameters analogous to that seen upon binding of α-lactalbumin to β-galactosyl transferase (28).

Chen et al. (29) have noted evidence suggesting that for a plasmid-encoded arsenical anion pump, binding of the ArsC protein to the pump complex either changes the specificity to arsenate from arsenite or increases the range of substrates to allow recognition of both arsenate and arsenite. No matter how the two transport proteins for cationic amino acids arise, a mechanism must exist to regulate the targeting of the activity of lysosomal system c to the lysosome, and that of system y+ to the plasma membrane.

Two major factors emerged from this study as playing important roles in determining the net direction and magnitude of cationic amino acid transport across the lysosomal membrane. The pH activity curve (Fig. 3) favors net uptake of cationic amino acids into the lysosome since transport is much faster away from the extralysosomal pH of 7.0 than it is away from the intralysosomal pH of 5.3. We demonstrated previously that MgATP accelerates lysine efflux 2-fold (8), and in this study, lysine and arginine uptake were both retarded severalfold when MgATP was present. Thus the effects of MgATP shift the equilibrium of the transport process in a manner favoring lysosomal egress of cationic amino acids. Smith et al. (16) have shown that significant changes in membrane potential occur on incubation of human fibroblast lysosomes with MgATP and have proposed that lysosomal egress of cystine is regulated by both the lysosomal membrane potential gradient and the transmembrane pH gradient. Jonas (30) has made similar observations regarding cystine exodus from rat liver lysosomes. Whether these or other possible changes are responsible for the effect of MgATP on lysosomal system c activity is not known at the present time. Under conditions of nutritional deprivation, proteolysis is shifted toward the lysosome. Perhaps this has significance with regard to nutritional survival of the cell such that the lysosome may act as a reservoir for amino acids with the net flux being regulated in part by the nutritional need of the cell.
A great diversity occurs in the structure of macromolecules delivered to the lysosome, some arising from the host organism but others arising from exogenous sources. Consequently, transport systems present in the lysosome may have evolved to mediate the flow of a broader spectrum of substrates than is encountered by the transport systems of the plasma membrane. We see this possibility in contrasting utilities of different transport systems for the purpose of drug utilization of mucopolysaccharide. Our results with thiocholine coenzyme sulfate within the lysosome or for possible metabolites for utilization within the lysosome or for possible signalling or information storage. Bame and Rome (31) have recently characterized a lysosomal membrane protein, acetyl coenzyme A: \( \alpha \)-glucosaminide N-acetyl transferase, which catalyzes the transfer of an acetyl group from cytosolic acetyl CoA to terminal \( \alpha \)-linked glucosamine residues of heparan sulfate within the lysosome. A deficiency of this enzyme, as occurs in Sanfilippo C syndrome, results in lysosomal accumulation of mucopolysaccharide. Our results with thiocholine emphasize the importance of knowing the substrate specificities of different transport systems for the purpose of drug design. The quaternary nitrogen of thiocholine renders the cationic product generated upon sulphhydril-disulfide exchange of thiocholine with cystine recognizable by lysosomal system c but not by system y+.

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