Characterization of Carrier-mediated Transport Systems for Small Neutral Amino Acids in Human Fibroblast Lysosomes

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* Received for publication, October 7, 1986

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Analog inhibition studies of the uptake of proline, serine, and threonine into human fibroblast lysosomes, purified on Percoll gradients, reveal the presence of three new transport systems. These systems fail to show the Na⁺ requirement usual for the plasma membrane. Proline uptake into fibroblast lysosomes occurs mainly by two routes: a predominant route half-saturating at 0.01 mM, and a lower-affinity route, half-saturating at 0.07 mM. The latter so far appears specific for L-proline and its 3,4-dehydro derivative. The high affinity route has a broad scope, recognizing best, beyond these two amino acids, various unbranched neutral amino acids not over 5 carbons long. Neither system accepts to a significant extent D-proline, hydroxyproline, cationic or anionic amino acids, nor neutral ones with bulky side chains. 2-Aminoisobutyrate and its N-methyl derivative have little effect on proline uptake, in contrast to their effectiveness on its uptake by the intact fibroblast. The rate of lysosomal proline uptake maximizes at about pH 6.4, is inversely related to the osmolality of the medium, and is unaffected by the extralysosomal presence of MgATP. The competition among alanine, serine, and threonine points to sharing of the broad-scope system for proline, although the main part of their uptake occurs by a third route that rejects amino acids in which the α-amino group is methylated.

Lysosomes are a major intracellular site for the degradation and processing of many kinds of macromolecules. Lysosomal protein degradation results in the formation of amino acids which were until recently thought to escape through holes in the lysosomal membrane. In the last 5 years, however, three lysosomal amino acid transport systems have been characterized and the question is raised how comprehensive is the set of systems participating. Gaith et al. (1-3) with human leucocytes and Jonas et al. (4) with human lymphoblasts demonstrated that a lysosomal carrier-mediated system transporting the amino acid, cystine, is defective in cystinosis. We have observed this cystine transport system as defective in cystinotic human fibroblasts and, in addition, have characterized a carrier-mediated transport system for cationic amino acids in the lysosomes of human fibroblasts, one which can apparently be used to serve therapeutically in cystinosis (5). Most recently, Bernar et al. (6) have described an amino acid transport system associated with lysosomes of rat thyroid FRTL-5 cells which is selective for bulky nonpolar neutral amino acids. Our search for further systems continues.

Studies with these previously characterized lysosomal transport systems were aided by utilizing associated trans-stimulation properties to characterize amino acid exodus or exchange from the lysosomes. Many of the amino acids, however, have not shown trans-stimulation properties associated with their release from the lysosome. Since transport systems are generally bidirectional, we have chosen to characterize lysosomal transport systems by studying the uptake of radiolabeled amino acids into purified lysosomes.

EXPERIMENTAL PROCEDURES

Normal human fetal fibroblasts (GM 0010) obtained from the Human Genetic Mutant Cell Repository were grown and maintained in 100-mm tissue culture dishes or 850-cm² roller bottles in Coons's modification of Ham's F-12 medium (KC Biological), supplemented with 10% fetal bovine serum. The granular fraction prepared as described previously (5) from human fibroblasts of 0.5-ml packed volume or less was placed on top of 10 ml of a 34.4% Percoll solution (288 mosm) in 18 mM MOPS/Tris pH 7.0 buffer containing 0.24 M mannitol and 1 mM Na₂EDTA and centrifuged for 35 min at 17,500 rpm in a Sorvall SS-34 rotor at 4 °C. The bottom third of the Percoll gradient, which is highly enriched in lysosomes, was diluted to 40 ml with 20 mM MOPS/Tris pH 7.0 buffer containing 0.275 M mannitol and 1 mg/ml bovine serum albumin (wash buffer) and the mixture centrifuged for 15 min at 20,000 × g in a Sorvall SS-34 rotor. The supernatant was discarded, the pellet portion transferred to a 1.5-ml microcentrifuge tube, diluted to 1.5 ml with the wash buffer, and washed twice by centrifugation for 12 min at 15,600 × g at 4 °C in an Eppendorf model 5414 microcentrifuge. The final lysosomal pellet was generally resuspended in 100 mM citrate/Tris pH 7.0 buffer.

For the analog inhibition and Ka experiments, 11.4-μl aliquots of an inhibitor or substrate solution in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 μM sucrose are mixed with an equal aliquot of radio-labeled amino acid in 0.25 mM sucrose and warmed to 37 °C. 11.4-μl aliquots of ice-cold lysosomal suspension in 100 mM citrate/Tris pH 7.0 buffer were added at time 0 and the mixtures incubated for 2.5 min at 37 °C, at which time a 25.5-μl aliquot was 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 scintillation fluid. Blanks were run for each experiment by substituting 100 mM citrate/Tris pH 7.0 buffer 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 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 (5) and was generally found to be 75-80%. Uptakes could therefore be expressed as

* This work has been supported by Grants DK33281 and DK25548 from the National Institutes of Health, United States Public Health Service. A preliminary report of this work has appeared in abstract form (Pisoni, R. L., Flickinger, K. S., Thoene, J. G., and Christensen, H. N. (1986) Fed. Proc. 45, 1759). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: MOPS, 3-(N-morpholino)propane-sulfonic acid; MBS, 2-(N-morpholino)ethanesulfonic acid.
as pmol of amino acid taken up during the indicated time period/unit of latent β-hexosaminidase activity. Uptake measured on this basis was found to decrease considerably as the cell passage number increased. As a consequence, cells with low passage numbers were used whenever possible and generally not beyond passage 22. For analog inhibition experiments, uptakes were performed in duplicate except for buffer controls which were run in triplicate.

β-Nucleotidase was measured by the method of Rome et al. (7) for 30 min at 37 °C. A unit is equivalent to 1 nmol of [3H]AMP hydrolyzed per min at 37 °C. β-Galactosyltransferase was measured by the method of Elices et al. (6) by counting the amount of [3H]galactose transferred to β-GlCNac-Synsorb beads from UDP-[3H]Gal and letting 1 unit represent 1 pmol of galactose transferred per min at 37 °C. Succinate dehydrogenase was assayed by reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride as described by Pennington (9). A unit of this enzyme is equivalent to 1 pmol of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride reduced per min at 37 °C.

Osmolarity measurements were performed with an Advanced Instruments freezing point depression osmometer.

Analysis of Data—The nonsaturable component of uptake was determined by secondary analysis of initial velocity kinetic data using the method of Elices et al. (6) by counting the amount of [3H]galactose transferred to β-GlCNac-Synsorb beads from UDP-[3H]Gal and letting 1 unit represent 1 pmol of galactose transferred per min at 37 °C. Succinate dehydrogenase was assayed by reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride as described by Pennington (9). A unit of this enzyme is equivalent to 1 pmol of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride reduced per min at 37 °C.

Results

The granular fraction from human fibroblasts when separated on a 34% Percoll gradient yields two peaks of lysosomal activity as judged by the distribution of latent β-hexosaminidase activity as a marker for intact lysosomes. Approximately 60% of the latent β-hexosaminidase activity is associated with a population of lysosomes sedimenting at densities between 1.07 and 1.13 g/cm3, the remaining being associated with a buoyant lysosomal population sedimenting at 1.035–1.05 g/cm3. Previous studies by others (7, 11, 12) have shown that the densely sedimenting lysosomal population from human fibroblasts is relatively free of enzyme activities specific for other intracellular organelles. In agreement, we found that the marker enzymes, 5'-nucleotidase for plasma membrane, succinate dehydrogenase for mitochondria, and β-galactosyltransferase for Golgi apparatus were present to a significant degree only in the buoyant fractions near the top of the Percoll gradient (data not shown). Consequently, only lysosomes from the bottom one-third of the Percoll gradient were used in the amino acid uptake studies described in this paper.

Amino acid methyl esters have been shown by Reeves (13) to specifically accumulate to high levels into lysosomes as compared with the other intracellular organelles. When loaded at high concentrations, some amino acid methyl esters will selectively cause the rupture of lysomes. When we exposed Percoll-purified lysosomal fraction to 2 mM L-proline methyl ester for 30 min at 25 °C, 80% of the lysosomes were found to have been lysed, compared to 35% in purified non-load control lysosomes as judged by their loss of β-hexosaminidase latency. A reduction in proline uptake comparable to the degree of lysis was observed from the proline methyl ester-treated lysosomes, suggesting that proline uptake is associated with intact lysosomes and not a contaminating organelle which might be present below our level of detection by marker enzyme assays.

A time course of [3H]L-proline uptake into Percoll-purified fibroblast lysosomes is shown in Fig. 1. The rate of proline uptake remains constant over the first 3 min of uptake, thereafter diminishing, but not reaching a steady state within 20 min of observation. In contrast to the fibroblast plasma membrane, lysosomal proline uptake occurs in the absence of added sodium ion. Moreover, no acceleration but a moderately reduced rate of lysosomal proline uptake is observed when 80 mM NaCl is present. The addition together of MgCl2 and Na2ATP had no effect on proline uptake.

The rate of proline uptake was found to vary inversely with the osmolarity of the suspending buffer, indicating that proline uptake occurs into intact lysosomal vesicular structures (Fig. 2). When the data are extrapolated to infinite osmolarity,

![Fig. 1. Effect of NaCl and MgATP on the time course of [3H]-proline uptake into Percoll-purified lysosomes isolated from human fibroblasts. Human fibroblast lysosomes were incubated at 37 °C with 0.038 mM [3H]-proline in 50 mM citrate/Tris pH 7.0 buffer containing either (a) 0.125 M sucrose, (b) 80 mM NaCl, or (c) 0.125 M sucrose plus 2 mM MgCl2 and 2 mM Na2ATP. At the indicated time points, aliquots were removed from the given incubation mixture, lysosomes were collected and washed on GF/A filters as described under "Experimental Procedures," and filters were counted for radioactivity.](image1)

![Fig. 2. Effect of osmolarity on [3H]-proline uptake into human fibroblast lysosomes. Human fibroblast lysosomes were incubated at 37 °C for 2.5 min with 0.038 mM [3H]-proline in 50 mM citrate/Tris pH 7.0 buffer to which sucrose of an appropriate concentration was added to obtain the desired osmolarity. Control uptake was performed in buffer containing 0.125 M sucrose (292 mosm) and resulted in 0.12 pmol of proline taken up during the incubation period. Shown is the percent of control uptake as a function of the inverse of the osmolarity of the incubation mixture.](image2)
Lysoisomal proline uptake shows a broad maximum in the neutral pH range, centered near pH 6.3, and declines significantly at pH values acidic to 6.0 or alkaline to 7.0 (Fig. 3).

A Michaelis-Menten plot of the velocity of lysosomal proline uptake as a function of substrate concentration demonstrates that the uptake process is saturable (Fig. 4A). The nonlinear nature of the Lineweaver-Burk plot (Fig. 4B) suggests, however, that proline uptake is not a homogeneous process but probably involves more than one pathway of uptake. The apparently biphasic nature of this lysosomal proline uptake as shown by the Eadie-Scatchard plot (Fig. 4C) allows at least two major routes of uptake to be calculated: a high affinity system for which the apparent $K_m$ is 0.01 mM, accounting for approximately 60% of the total proline uptake, and a lower affinity system for which it shows an apparent $K_m$ of 0.07 mM serving for the remainder of the mediated proline uptake.

General characteristics of these lysosomal transport systems serving for proline uptake are displayed in Table I in which various amino acids, each at a concentration of 6.7 mM, were tested for their ability to inhibit 0.05 mM $[^{14}C]L$-proline uptake. It is seen that the D-isomer of proline, either very little effect on lysosomal proline uptake. In addition, evidenced by the equal effectiveness of sarcosine and glycine. were tested for their ability to inhibit 0.05 mM $[^{14}C]L$-proline uptake into fibroblast lysosomes.

As shown in Fig. 5, A-C, alanine inhibits 0.03 mM $[^{14}C]L$-proline uptake by approximately 70%. This level of inhibition, obtained at a relatively low alanine concentration, is not increased substantially beyond this level if the alanine concentration is increased to 6.7 mM (Table I). This result further supports lysosomal proline uptake occurring by at least two different routes of mediation: an alanine-sensitive route accounting for the majority of the total proline uptake and one or more alanine-insensitive routes comprising the remainder of the total proline uptake. These two appear to correspond to the two major routes shown by the kinetic analysis of Fig. 4C.

When lysosomal proline uptake is studied in the presence of 1 mM L-alanine (Fig. 6), much of the uptake by the high affinity system is lost with uptake by the lower affinity system of an apparent $K_m$ of 0.07 mM predominating to the extent of 70% of the total uptake. These results suggest that of the two major routes of lysosomal proline uptake, the high affinity system of apparent $K_m$ of 0.01 mM is the alanine-sensitive route serving for the transport of both alanine and proline, whereas the lower affinity system appears to exclude alanine.

The ability of various amino acids to inhibit proline transport in the presence of a saturating concentration of alanine was tested to determine if these other amino acids share the same high affinity proline transport system as does alanine or are recognized by the residual lower affinity system of
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100

ao

a-amino n-butyrate
L-Alanine

A

D-Proline Norvaline

lnhibitor,mM

100

100

Valine Glycine N-Methyl Ala Sarcosine L-Alanine

B

[Per Cent of Uninhibited Rate]

[Inhibitor],mM

[Per Cent of Uninhibited Rate]

[Inhibitor],mM

[Per Cent of Uninhibited Rate]

[Inhibitor],mM

FIG. 5. Concentration dependence of inhibition of proline uptake into human fibroblast lysosomes by various amino acids. The uptake of 0.05 mM [3H]proline into fibroblast lysosomes was measured in the presence of various amino acids at concentrations ranging from 0 to 3.0 mM in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 M sucrose. Results are from several experiments with 2–4 determinations performed for each amino acid shown and are expressed as percent of uninhibited control which were usually in the range 0.5–1.0 pmol of Pro/hexosaminidase.

FIG. 6. Kinetics of lysosomal proline uptake in the presence of 1 mM L-alanine. Fibroblast lysosomes were incubated for 2.5 min at 37°C with [3H]proline of the indicated concentration in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 M sucrose and 1 mM L-alanine. At the completion of the incubation period, lysosomes were collected and washed on GF/A filters, and the amount of proline taken up was determined by counting radioactivity retained on the filter. Data is shown in an Eadie-Scatchard analysis, and a nonsaturable component of $K_d = 0.25$ pmol proline/hexosaminidase (hex)$^{-1}$ min$^{-1}$ mM$^{-1}$ has been subtracted from the data.

None of the tested amino acids in the presence of alanine was able to reach the level of inhibition obtained with L-proline. Instead, all amino acids in the presence of alanine inhibited proline uptake to approximately the same degree as that obtained with alanine alone. Thus, the effect of these amino acids on lysosomal proline uptake appears to be limited to the high affinity alanine-sensitive route of mediation, whereas the residual lower affinity route of proline uptake appears to serve exclusively for L-proline and, as shown later, possibly 3,4-dehydro-L-proline.

Many of the small neutral amino acids tested in Table I proline mediation (Table II). None of the tested amino acids in the presence of alanine was able to reach the level of inhibition obtained with L-proline. Instead, all amino acids in the presence of alanine inhibited proline uptake to approximately the same degree as that obtained with alanine alone. Thus, the effect of these amino acids on lysosomal proline uptake appears to be limited to the high affinity alanine-sensitive route of mediation, whereas the residual lower affinity route of proline uptake appears to serve exclusively for L-proline and, as shown later, possibly 3,4-dehydro-L-proline.

Many of the small neutral amino acids tested in Table I were served in the uninhibited control for the different experiments were: A, 0.23 pmol/hexosaminidase; B, 0.39 pmol/hexosaminidase; and C, 0.29 pmol/hexosaminidase.

### Table I

| Inhibition of 0.05 mM [3H]l-proline uptake into fibroblast lysosomes by various amino acid analogs |
|---|
| L-Proline & 4 ± 2<br>trans-Hydroxy-L-proline & 68 ± 7<br>3,4-Dehydro-L-proline & 13 ± 3<br> cis-Hydroxy-L-proline & 72<br>L-Alanine & 30 ± 5<br>2-Aminoisobutyrate & 72 ± 2<br>β-Chloro-L-alanine & 28 ± 2<br>2-(Methylamino)isoobutyrate & 87 ± 4<br>L-Serine & 35 ± 3<br>L-Proline & 35 ± 3<br>β-Alanine & 38 ± 6<br>1-Pyrrolidone & 85 ± 8<br>L-Diaminopropionate & 40 ± 2<br>l-Serine & 91 ± 5<br>L-Homoserine & 40 ± 5<br>Taurine & 97 ± 2<br>L-Pipecolate & 41 ± 3<br>1-Norleucine & 82 ± 1<br>N-Methyl-L-alanine & 43 ± 3<br>1-Methionine & 100 ± 7<br>3-Aminoisoobutyrate & 45 ± 2<br>1-Leucine & 95 ± 9<br>Sarcosine & 45 ± 2<br>1-Phenylalanine & 100 ± 6<br>L-Valine & 48 ± 2<br>1-Arginine & 90 ± 4<br>Glycine & 51 ± 3<br>1-Glutamate & 89 ± 3<br>L-Threonine & 51 ± 3<br>1-Aspartate & 85 ± 4<br>L-Isoleucine & 68 ± 2<br>N,N-Dimethylglycine & 102 ± 8<br>D-Proline & 68 ± 9<br>Betaine & 100 ± 4 |

| Inhibitor, mM |
|---|
| Table I |

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FIG. 5. Concentration dependence of inhibition of proline uptake into human fibroblast lysosomes by various amino acids. The uptake of 0.03 mM [3H]proline into lysosomes was measured for 2.5 min at 37°C in the presence of various amino acids at concentrations ranging from 0 to 3.0 mM in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 M sucrose. Results are from several experiments with 2–4 determinations performed for each amino acid shown and are expressed as percent of uninhibited control which were usually in the range 0.5–1.0 pmol of Pro/hexosaminidase.

FIG. 6. Kinetics of lysosomal proline uptake in the presence of 1 mM L-alanine. Fibroblast lysosomes were incubated for 2.5 min at 37°C with [3H]L-proline of the indicated concentration in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 M sucrose and 1 mM L-alanine. At the completion of the incubation period, lysosomes were collected and washed on GF/A filters, and the amount of proline taken up was determined by counting radioactivity retained on the filter. Data is shown in an Eadie-Scatchard analysis, and a nonsaturable component of $K_d = 0.25$ pmol proline/hexosaminidase (hex)$^{-1}$ min$^{-1}$ mM$^{-1}$ has been subtracted from the data.
TABLE II
Inhibition by selected amino acids of lysosomal proline uptake persisting in the presence of alanine

The ability of the indicated amino acids (2.5 mM in Experiment A and 11.8 mM in Experiment B) to inhibit lysosomal uptake of \[^{14}C\]L-proline (0.03 mM in Experiment A and 0.1 mM in Experiment B) was measured in the presence or absence of L-alanine (1.22 mM in Experiment A and 5.4 mM in Experiment B). Uptakes were for 2.5 min at 37 °C in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 M sucrose. Proline uptake in the uninhibited control was 0.311 pmol/ml fibroblast lysosomes in Experiment A and 0.73 pmol/ml/hexosaminidase in Experiment B during the uptake period. Results are given as the percentage of these control uptakes performed in the absence of any inhibitors.

| Inhibitor, 2.5 mM | Uptake observed | % of uninhibited rate |
|-------------------|-----------------|----------------------|
| N-Methyl-L-alanine | 45 ± 2          | 28 ± 2               |
| L-α-Amino-n-butyrate | 43 ± 2        | 40 ± 2               |
| L-Serine          | 42 ± 2          | 43 ± 2               |
| L-Pipecolate      | 50 ± 2          | 25 ± 2               |
| trans-Hydroxy-L-proline | 77 ± 6      | 26 ± 0               |
| Azetidine-2-carboxylate | 49 ± 2      | 34 ± 1               |
| L-Proline         | 35 ± 2          | 10 ± 1               |
| L-Alanine         | 32 ± 1          |                      |

| Inhibitor, 11.8 mM | Uptake observed | % of uninhibited rate |
|-------------------|-----------------|----------------------|
| N-Methyl-L-alanine | 43 ± 4          | 33 ± 2               |
| L-α-Amino-n-butyrate | 43 ± 4        | 40 ± 4               |
| L-Serine          | 45 ± 4          | 43 ± 7               |
| L-Pipecolate      | 53 ± 3          | 48 ± 4               |
| trans-Hydroxy-L-proline | 85 ± 5      | 37 ± 1               |
| Azetidine-2-carboxylate | 45 ± 3      | 37 ± 1               |
| L-Proline         | 22 ± 2          | 32 ± 2               |
| L-Alanine         | 40 ± 2          |                      |

Sensitivity of uptake of 0.03 mM L-proline to other amino acids persisting in the presence of 1.22 mM L-alanine

Sensitivity of uptake of 0.1 mM L-proline to other amino acids persisting in the presence of 5.4 mM L-alanine

The finding that lysosomal proline uptake is inhibited significantly by alanine and serine led us to study whether the lysosomal uptakes of these latter two amino acids are sensitive to inhibition by proline as a means of establishing shared transport. A time course of \[^{14}C\]L-alanine uptake by fibroblast lysosomes (Fig. 7) indicates that its uptake, like that of proline, occurs readily in the absence of added sodium ion and is moderately slowed rather than accelerated when 80 mM NaCl is present. Selected amino acids, each at a concentration of 6.7 mM, were examined for their ability to inhibit lysosomal uptake of \[^{14}C\]L-alanine, \[^{14}C\]L-serine, and \[^{14}C\]L-threonine. It was found that alanine, serine, and threonine strongly inhibited the uptakes of each other. The amino acids may share a common transport system. A proline-inhibited component of alanine and serine uptake is observed, consistent with their competition for a shared route, whereas 3-aminoisobutyrate and L-serine are less effective. As the side chain is lengthened in the case of homoserine or becomes branched as occurs with threonine, an even lower affinity for inhibiting lysosomal proline uptake is observed.

Further substantiation of this view is gained by reference to Table I where it is seen that any amino acid in which the linear side chain is more than 3 carbons in length results in no significant inhibition of lysosomal proline uptake. In Fig. 5B glycine, which has no side chain, and valine, in which the side chain is branched, both inhibit proline uptake but with a much lower affinity than does alanine. The presence of a side chain, 1 or 2 carbons in length, may enhance the efficiency of binding to the transport protein receptor site, but efficiency is significantly reduced upon introduction of a branch in the side chain. The ineffectiveness of trans- or cis-hydroxy-L-proline in inhibiting lysosomal proline uptake (Table I) may in part be explained by the side chain branch produced by the presence of the hydroxyl group. N-Methyl-L-alanine, which gives 65% inhibition at a concentration of 3 mM in Fig. 5B, is recognized by a high affinity proline transport system but with a much lower affinity than is alanine. We have noted a similar result in comparing the concentration curves of N-methyl-β-alanine and β-alanine as to the effect of N-methylation on the ability to inhibit proline uptake (data not shown). N-Methylation appears to have little effect in the case of sarcosine, however, which is slightly more effective than glycine in inhibiting lysosomal proline uptake. Finally, in Fig. 5C, β-chloro-L-alanine demonstrates the same high affinity for inhibiting proline transport as does L-alanine, whereas 3-aminoisobutyrate and L-serine are less effective. As the side chain is lengthened in the case of homoserine or becomes branched as occurs with threonine, an even lower ability to inhibit lysosomal proline uptake is observed.

Additional comparisons provide further support for this view.

![Fig. 7. Time course of \[^{14}C\]L-alanine uptake into Percoll-purified fibroblast lysosomes. Human fibroblast lysosomes were incubated with 0.08 mM \[^{14}C\]L-alanine in 50 mM citrate/Tris pH 7.0 buffer containing either (a) 0.25 M sucrose or (b) 0.083 M sucrose and 100 mM NaCl. At the indicated time points, aliquots of the incubation mixture were removed, lysosomes were collected and washed on GF/A filters, and the amount of radioactive alanine taken up was measured.](image)
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Amino Acid Uptake Observed

FIG. 8. Inhibition by selected acids of the lysosomal uptake of either 0.08 mM [14C]L-alanine, 0.08 mM [14C]L-serine, or 0.06 mM [14C]L-threonine. Uptakes were for 1.5 min at 37°C in 50 mM citrate/Tris pH 7.0 buffer containing 0.125 M sucrose. The inhibitor concentrations were 6.7 mM, and the results are shown as a percentage of the control uptake performed in the absence of inhibitor. Control uptakes during the incubation period were 0.41 pmol/hexosaminidase for alanine, 0.69 pmol/hexosaminidase for serine, and 0.80 pmol/hexosaminidase for threonine. MeAIB, 2-(methylamino)isobutyric acid.

1) Whereas leucine has been shown not to affect lysosomal [14C]L-proline uptake (Table I), the uptake of [14C]L-alanine, [14C]L-serine, and [14C]L-threonine are significantly inhibited by leucine. 2) L-Alanine is a strong inhibitor of its own uptake and the uptake of [14C]L-threonine, but N-methyl-L-alanine is a very poor inhibitor of [14C]L-alanine or [14C]L-threonine lysosomal uptake (Table III). In contrast, N-methyl-L-alanine strongly inhibits [14C]L-proline uptake and nearly to the same extent which L-alanine inhibits [14C]L-proline uptake (Table III). 3) Whereas glycine and sarcosine have each been shown to inhibit proline uptake by nearly 60%, glycine inhibits alanine uptake by 70%, but sarcosine produces only a 20% inhibition (data not shown). These observations strongly suggest that alanine, serine, and threonine enter fibroblast lysosomes by at least two systems, a major route of mediation in which N-methylated amino acids are not tolerated and a minor route in which transport is shared with small neutral amino acids in which the α-amino group is secondary as in proline, N-methyl-L-alanine, and sarcosine. Of course the possible participation of still other components in the uptake of alanine is not excluded.

DISCUSSION

The present study applies the widely used analog inhibition approach (15–17) for characterizing amino acid transport systems to purified lysosomes from human fibroblasts. Associated trans-stimulation properties have been employed in previous studies to determine the specificities of the known lysosomal amino acid transport systems for cystine (3), for the cationic amino acids (5), and most recently for bulky nonpolar neutral amino acids (6). The analog inhibition approach allows the characterization of transport systems which do not demonstrate trans effects and in general greatly enhances the ability to determine kinetic parameters of transport, the existence of heterogeneity involved in the transport of any given amino acid, and the substrate specificities for the different transport agencies.

As shown with some transport systems (18–20), the kinetic parameters determined on one side of the membrane may differ from those observed on the other side of the membrane.

TABLE III
Inhibition of alanine and threonine uptake into lysosomes of human fibroblasts by selected amino acids

| Inhibitor, 6.7 mM | [14C]L-Alanine uptake, 0.08 mM | % of uninhibited rate | [14C]L-Threonine uptake, 0.06 mM | % of uninhibited rate |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| None            | 100             | 100             |                  |                  |
| L-Alanine       | 11              | 20              |                  |                  |
| N-Methyl-L-alanine | 83            | 105             |                  |                  |
| L-Leucine       | 43              | 31              |                  |                  |
| L-Leucine + N-methyl-L-alanine | 33            | 34              |                  |                  |
Lysosomal Transport of Small Neutral Amino Acids

It is especially important to consider this aspect in the case of the lysosome in which the pH and membrane potential are significantly different on the inner and outer side of the lysosomal membrane. Christensen (21, 22) points to the situation for some small neutral amino acids in which net uptake into the Ehrlich cell occurs largely by the highly concentrative transport system A, whereas the less concentrative system L appears to operate for net exodus of these amino acids. Likewise, in the lysosome in instances when multiple pathways describe the total uptake of an amino acid, the major pathway serving for net uptake may prove not to be the predominant route of mediation for net exodus. It is a widely accepted concept that transport systems must on thermodynamic grounds be susceptible to reversal. Otherwise an uphill operation could result that exceeds the energy available to that system. Numerous transport systems demonstrate their reversibility by showing the phenomenon of trans-stimulation by analogs, where the flux in a given direction yields the same analog inhibition pattern for the cis flux as it does for stimulation of the trans flux. Because the characteristic of trans-stimulation has not been observed for the transport systems reported here, the evidence for their operation for release of the same amino acids from the functioning lysosome remains formally incomplete. The biological significance of uptake of various amino acids by lysosomes remains to be evaluated. Harm et al. (23) have found that for anionic and small neutral amino acids their lysosomal concentrations were lower than their respective concentrations in unfractionated rat liver. In contrast, for most of the remaining amino acids, their lysosomal concentrations exceeded their concentrations in unfractionated rat liver. The lysosomal concentration of each free amino acid presumably depends on its rate of formation within the lysosomal compartment and the difference in its rates of entry and exodus.

The analog inhibition strategy, employing radiolabeled proline and alanine as test substrates, has revealed the existence of three new lysosomal transport systems in the human fibroblast. These three new systems, shown in the schematic diagram of Fig. 9, we have provisionally designated lysosomal systems e, f, and p.2 Lysosomal system p corresponds to the low affinity proline transport component which appears so far to be highly specific for L-proline and possibly 3,4-dehydro-L-proline. Lysosomal system f has been proposed for the broader scope high affinity lysosomal proline transport system. This route of mediation is similar to system A present in the plasma membrane of various cells (15, 25, 26), including the human fibroblast (14), both systems being selective for small neutral amino acids, including those in which the α-amino group is methylated, and both serving for the major portion of proline transport. These two systems bear, however, some important differences. In addition to differences in their Na+ requirement, 2-(methylamino)isobutyric acid, which strongly inhibits system A, has a weak inhibitory effect on lysosomal system f. Also the pH sensitivity for proline transport differs between the lysosome and plasma membrane (15, 26, 27), and some β-amino acids are recognized almost as well as their α-amino acid counterparts by lysosomal system f (Table I, Fig. 5C) but not by plasma membrane system A (28). The difference in sensitivity to 2-(methylamino)isobutyric acid between the plasma membrane and the lysosomal membrane may result from an unfavorable stereochemistry for binding at the lysosomal transport sites arising from the excessive branching at the α-carbon, since α-aminoisobutyrate is also ineffective as an inhibitor of proline uptake.

Christensen et al. (29) initially demonstrated in the Ehrlich cell and later in many other cell types (26, 30, 31) that system ASC is selective for small neutral amino acids, but in contrast to system A, amino acids in which the α-amino group is methylated are poorly recognized. Although alanine is recognized by both systems A and ASC, Gazzola et al. (14) found that most carrier-mediated transport of alanine across the plasma membrane of the human fibroblast occurs by system ASC. Our studies (Fig. 8 and Table III) indicate the existence of at least one transport system in the lysosomal membrane, which, similar to ASC, serves as the major route for the passage of alanine, serine, and threonine and does not tolerate amino acids in which the α-amino group is methylated. This latter lysosomal amino acid transport system we designate as lysosomal system e.

Over the years model amino acids whose mediation is confined to a specific transport system have greatly aided the ability to discriminate among the various transport agencies (15, 25, 32–39). N-Methyl-L-alanine or sarcosine appear to be good candidates as model amino acids for lysosomal system f, whereas lysosomal systems p and e require additional investigation to define more clearly their substrate specificities.

The striking effect of osmolarity on proline transport leads one to consider to what degree intralysosomal activities are regulated by osmolarity. In cystinosis and other lysosomal storage disorders, the accumulation of a metabolite would be

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2 We are constantly reminded of the need for brief designations for added amino acid transport systems, especially when these ultimately call for frequent citation and when the temptation exists, for example, to regard one of them erroneously as one more occurrence of a known system. Descriptions in words of each of the three new systems shown here, beginning in each case with the adjective lysosomal, are perhaps ideal, but may soon prove bulky. Furthermore, we suspect on genetic grounds that none of the systems so far described for the lysosome is likely to prove identical with one expressed in the plasma membrane. Already we begin to regret having used provisionally the designation y+ for System y+ for any known lysosomal system; the designation for System p has been permitted to be evocative because of its apparently high specificity to proline. Wherever such designations seem justified for economy of space, we regard the term lysosomal as a necessary part. The letters are lower case because no Na+ dependence is so far recognizable.
expected to be accompanied by increased water migration into the lysosome to maintain the osmolarity with that of the extralysosomal medium. This increase of the intralysosomal volume could diminish the catalytic rate of those intralysosomal enzymes or transport proteins in which lowering of the substrate concentration, as a result of dilution, occurs at a sensitive portion of the substrate concentration curve of the given catalytic protein.

Given that work continues to verify that amino acids are transported by different systems in the lysosome as opposed to the plasma membrane, then certain consequences are apparent: presumably these systems are under separate genetic regulation, and a targeting mechanism to sort the proteins to the plasma and lysosomal membranes is required. Elucidation of the presumed targeting mechanism will further enhance understanding of how intracellular protein traffic is directed.

Acknowledgment—We especially wish to express our gratitude and appreciation to Dr. Bruce Donohoe of the Department of Anatomy and Cell Biology of the University of Michigan for the use of the Advanced Instruments freezing point depression osmometer.

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