Characterization of the Lysosomal Cystine Transport System in Mouse L-929 Fibroblasts*

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We characterize here a lysosomal cystine transporter in mouse L-929 fibroblasts. Granular fractions from cells preloaded with cystine demonstrated counter-transport that showed no dependence on Na+ or K+. The Michaelis constant for infinite-trans influx was 0.27 ± 0.06 mM (n = 3), and a nonsaturable component of cystine entry was observed with Kd = 0.8–1.8 nmol of cystine·min−1·unit of hexosaminidase−1·mM−1. We found no evidence that cystine was also carried on any of the other known lysosomal amino acid transporters. Over 50 analogs were tested for their ability to inhibit countertransport. The inhibition constants are reported for selenocysteine, cystathionine, selenomethionine, and leucine. Significant requirements for recognition by the transporter were the presence of amino groups, t configuration, and a chain length not greater than eight atoms. A net positive or negative charge was not required. Some di- as well as tetrapolar amino acids were recognized. We have surmised that the binding site has polar and apolar domains, the latter being large enough to accommodate branching on C-3 and the substitution of selenium or carbon in place of sulfur.

Lysosomes are a major site for intracellular degradation of many types of macromolecules including proteins. The first realization that lysosomes have specific transport systems for amino acids derived from the demonstration that the accumulation of cystine found in the inherited metabolic disease, cystinosis, was the result of the absence of a functional lysosomal transport system for cystine (1–3).

Cystine transport in normal lysosomes can be studied experimentally by raising the internal cystine concentration by means of cystine dimethyl ester (CDME)† (3–8). Studies in this laboratory showed that the exodus of cystine from granular fractions or Percoll-purified lysosomes of normal human lymphoblasts, leukocytes, and fibroblasts proceeded with a first-order dependence on the initial intralysosomal cystine load. At these subsaturating concentrations, exodus was stimulated by the addition of Mg/ATP, by an increase in the transmembrane pH gradient, and by a decrease in the transmembrane potential (8). Only in leukocytes was it possible to load with sufficient cystine to saturate the internal binding site, a concentration at which the response to Mg/ATP disappeared (9). Countertransport has been used by Gahl et al. (5) to characterize the lysosomal cystine transport system in the granular fraction of human leukocytes. These studies (5) established the presence of a carrier mediated system, provided an estimate of 0.5 mM cystine for the Kd0 of the external binding site, showed a preference for L-cystine and competition by cystathionine, cystamine, and cysteamine-L-cysteine mixed disulfide. Countertransport of cystine has also been demonstrated in FRTL-5 rat thyroid epithelial cells (10).

In this study we have taken advantage of the properties of mouse L-929 fibroblasts for further characterization of the lysosomal cystine transport system. The lysosomes of this cell type allow internal loads of cystine sufficiently high to saturate the internal binding site of the carrier. As a result, we have been able to make studies of cystine countertransport which allow us to test the recognition of the external binding site for other amino acids and various analogs of cysteine. This, in turn, enables us to deduce whether the cystine transporter is similar to other lysosomal amino acid transporters and what chemical structures the transporter accepts for binding.

In addition, unlike leukocytes, L-929 fibroblasts can be grown in culture in large quantities. This approach has enabled us to perform kinetic studies that reveal a nonsaturable component. In addition, these studies provide the basis for developing a binding site inhibitor and for understanding the vestigial cystine transport capacity of some cystinosis types.

EXPERIMENTAL PROCEDURES

Materials—L-[4-14C]Cystine was obtained from Du Pont-New England Nuclear (307 mCi/mM). We found that this product contained impurities of 1.4–1.7% cysteic acid and 0.3% cysteine sulfenic acid, levels that were constant over the period of use. BCH, 1,4-diaminocarbonylcyclohexane, and L-3-methylvaline (tart-leucine) were gifts of Dr. Halvor Christensen, Dept. of Biological Chemistry, University of Michigan, Ann Arbor; N5-acetyl ornithine was the gift of Dr. John Thompson and Dr. Stephen P. F. Miller, NIH; L-, D-, and meso dianimopimolopimol, L-(3R) hydroxydianimopimolopimol, LL-, DD-, and meso-4-exo-methylene-dianimopimol, and LL- and DD-lanthionine were gifts from Dr. John C. Vederas, Dept. of Chemistry, University of Alberta, Edmonton, Alberta, Canada; L-aspartate, L-selenomethionine, L-leucine, and D-valine were obtained from Calbiochem. L-Djenkolate was from U. S. Biochemical Corp., Cleveland, OH, and S-carboxyethyl-L-cysteine was from Adams Chemical Co., Round Lake, IL. All other test compounds were obtained from Sigma. Other chemicals were the purest available from commercial sources.

Tissue culture media, trypsin, and penicillin/streptomycin were obtained from Irvine Scientific, Santa Ana, CA; calf serum and fetal...
bovine serum were from Gemini Bio-Products Inc., Calabasas, CA. Substrate for the \( \beta \)-hexosaminidase assay (4-methylumbiferyl \( \beta \)-d-glucopyranoside) was purchased from Koch Light Laboratories Ltd., Colnbrook, Berkshire, United Kingdom.

**Cell Culture:** Stock cells of an L-929 mouse fibroblast clone were maintained on 100-mm plates in minimum essential medium with 10% serum (3, calf serum; fetal bovine serum) plus fresh glutamine. Cells were grown in 85% plastic roller bottles in minimum essential medium, 10% fetal bovine serum, 50 units/ml penicillin, 50 \( \mu \)g/ml streptomycin sulfate, and 5\% CO\(_2\). A culture reaching passage 30 was replaced by freshly thawed cells because older cultures showed a decline in yield of granular fraction.

**Collector and Cystine Loading of Cells:** Cells were harvested by trypsinization as detailed previously (8, 11). Cells were loaded by incubation for 20 min at 37 \(^\circ\)C with 1.5 mM CDME and 20 mM Heps, pH 7, at a density of 1.5 \( \times \) 10\(^6\) cells/ml in serum-free Coon's F-12 medium made without cystine. Cells were repelted, washed twice with ice-cold phosphate-buffered saline, and once with cold 0.25 M sucrose. The value achieved for nmol of cystine/unit of \( \beta \)-hexosaminidase varied with the preparation despite constant conditions for loading.

**Isolation of Granular Fraction:** The granular fraction was prepared by lysis of the cells in cold 0.25 M sucrose by means of repeated pipetting through a 1-ml disposable pipette tip affixed to the end of a 10-ml disposable pipette. When microscopically examined showed that an equal volume of 0.25 M sucrose was added, and the suspension was pelleted at 1,000 \( \times \) g for 10 min in a refrigerated centrifuge. The supernatant was saved and the pellet resuspended and pipetted until approximately 80% of the cells were lysed, and then it was centrifuged. The combined supernatants were pelleted at 20,000 \( \times \) g for 10 min. The granular fraction was washed in Hepes/sucrose buffer (20 mM Hepes, pH 7, 0.25 M sucrose) and then resuspended for use in experiments. Any preparation in which the latency of \( \beta \)-hexosaminidase was less than 80% was not used.

**Assays:** Cystine determinations were performed using a binding assay (12). Rates and concentrations were determined spectrophotometrically (14), and \( \beta \)-hexosaminidase activity was determined fluorometrically (13, 15). A unit of \( \beta \)-hexosaminidase activity is the amount of enzyme that will hydrolyze 1 pmol of substrate/min. Latency was evaluated by comparing the \( \beta \)-hexosaminidase assay in both an isotonic buffer and a hypotonic buffer containing 1% Triton X-100.

**Countertransport:** The rate of radioactive cystine uptake was measured by sampling from the test suspension during its incubation at 37 \(^\circ\)C. The suspension contained an aliquot of the stock granular fraction, 1 mM NEM, 8 \( \mu \)M \[^{35}\text{S}\]cystine, Heps/sucrose buffer, pH 7, and the test compound or equivalent control. The final mixture was always 363 mM NaCl. Samples were taken at three time points (0-3 min) and each was delivered into 1 ml of cold Heps/sucrose buffer. The diluted sample was pulled through a CF/C glass microfibre filter followed by two 10-ml portions of ice-cold phosphate-buffered saline. The filters were dried, the radioactivity measured by liquid scintillation counting, and the counts converted to pmol of cystine/unit of \( \beta \)-hexosaminidase based on the specific activity of the labelled cystine and the assay of \( \beta \)-hexosaminidase in the stock suspension. The rate of cystine uptake was determined by linear regression analysis. Latency, evaluated at the end of the incubations in the presence of many of the test compounds and especially for those used at high concentrations, did not change during the 3-min incubation; therefore, no corrections for changes in latency were made. Rates in the presence of competitors shown in Tables IV and V are expressed as percent of the uninhibited control rate for which the mean and S.E. were 60 \( \pm \) 2 (n = 27) pmol of cystine\(\cdot\)min\(^{-1}\)\cdot\)unit of hexosaminidase\(^{-1}\). Since duplicate incubations of either inhibited or uninhibited control samples yielded uptake values within \( \pm \) 5% of the mean during the course of experiments on a given day, the ratio of sample to control would be expected to vary within approximately \( \pm \) 5% of the means that are reported in Tables IV and V. (The square of the quotient percent error equals the sum of the squares of percent error of the numerator and denominator.)

**Kinetics—Mediated membrane transport may be described by the two unit reaction sequence (16, 17) wherein the substrate (S) binds to an active site on side 1 of the membrane followed by the ionization of carrier/substrate to release it on side 2. In our experiments, \( S_1 \) is radioactive cystine (\[^{35}\text{S}\]cystine) in the exterior reaction volume, and \( S_2 \) is the interior substrate, nonradioactive cystine generated from CDME loading. In countertransport experiments the rapid exodus and release of unlabeled cystine increase the availability of empty carriers on the external side so that the uptake of labeled cystine is stimulated. The rate of this trans-stimulated uptake is dependent on substrate concentrations on both sides of the membrane and may be described by parameters typical of a saturable transport system. As \( S_1 \) increases from zero to infinite-trans (saturating) conditions, the Michaelis constant for influx (\( K_m \)) increases somewhat, but the observed \( V_{\text{max}} \) for influx increases severalfold as expected with trans-stimulation. When the interior concentration is saturating, \( V_{\text{max}} \) becomes maximal and the ratio of \( V_{\text{max}} \) to \( K_m \) all become maximum, and the descriptive equation becomes

\[
v = \frac{V_{\text{max}} [S_1]/(K_2 + [S_1]) + [S_2]}{K_1}
\]

where \( v \) is the initial rate, \( V_{\text{max}} [S_1]/(K_2 + [S_1]) + [S_2] \) is the steady-state rate for the carrier saturated both for entry and exit of substrate; \( [S_1] \), concentration of radiolabeled exterior substrate; and \( K_1 \), the Michaelis constant for infinite-trans influx. The notation is that used by White and Christensen (17). This expression may be expanded to include a nonsaturable component as follows

\[
v = \frac{V_{\text{max}} [S_1]/(K_2 + [S_1]) + [S_2]}{K_1} + \text{nonsaturable flux}
\]

where \( K_1 \) is the first-order rate constant for nonsaturable influx. The experimental data were test fitted to each of the above equations by nonlinear least squares analysis using Enzfitter (R. J. Leatherbarrow, from Biosoft, Cambridge, U. K.).

**RESULTS AND DISCUSSION**

**Countertransport into Granular Fractions—Mouse L-929 fibroblasts have a specific lysosomal transport system for cystine and, like human leukocytes, show countertransport that is demonstrable through the uptake of L-[\(^{35}\text{S}\)]cystine into cystine-loaded granular fractions. Conditions were established so that the rate of uptake over the first 3 min was linear and proportional to the amount of granular fraction taken (Fig. 1).

Experiments shown in Table I establish that the countertransport requires intact vesicles, physiological temperatures, and that external 50 mM NaCl or KCl has no effect. Although cystine uptake decreases as expected with increasing osmolality of the suspending medium, small changes in osmolality such as might arise from unavoidable differences in the incubation mixtures containing competitors have negligible effect (data not shown).

The rate of trans-stimulated radioactive cystine uptake will be maximal when the lysosomal interior binding site is saturated with unlabeled cystine (16, 17). We have measured the relation between interior cystine load and rate of cystine countertransport using granular fractions from a preparation of cells loaded to different levels. The relation is shown in Fig. 2. The solid curve was generated by a least squares fit of

![Fig. 1. Countertransport in L-929 cell granular fractions.](http://www.jbc.org/)
lysosomal cystine transport

TABLE I

Effect of sonication, Triton, cold, and salts on countertransport of cystine uptake by mouse L-929 granular fraction

| Treatment | Rate | Load |
|-----------|------|------|
|           | pmol cystine·min⁻¹·unit hexosaminidase⁻¹ | nmol cystine·unit hexosaminidase⁻¹ |
|           | pmol of cystine·unit of hexosaminidase⁻¹·min⁻¹ |
| Exp. 1    | 6.3  | 10   | 59  |
| Standard  | 0.2  | 0.2  |     |
| Exp. 2    | 6.1  | 17.5 | 43  |
| Standard  | 1.8  | 9.1  |     |
| Exp. 3    | 6.4  | 35   | 120 |
| Standard  | 4.2  | 4    |     |
| Exp. 4    | 6.9  | 50.3 | 93  |
| Standard  | ND   | 48.3 |     |
| +50 mM NaCl | ND | 50.3 |     |
| +50 mM KCl | ND  | 50.3 |     |

a Standard treatment was incubation of granular fraction at 37 °C in 1 mM NEM, 8 μM L-[¹⁴C]cystine, 20 mM Na⁺-Hepes, pH 7, and 0.25 mM sucrose.

b 50 mM salts and 150 mM sucrose instead of 250 mM sucrose in incubation mixture.

c ND, not determined.

The data to a rectangular hyperbola of the Michaelis form using the explicit weighting dictated by the error bars. This curve is an approximation because the exact form of the equation (equation 6 of White and Christensen (17) or equation IX-56 of Segel (16)) which describes countertransport at partial saturation requires values for parameters unavailable to us. The same experiment performed on another day shows the same K₉₅ for internal saturation but a different maximum rate. Such day-to-day variation has been observed by others (18).

Kinetics—Initial rates of countertransport versus external substrate concentration were measured in a preparation loaded to saturation and analyzed as described under “Experimental Procedures.” The data were fitted both to the simple Michaelis equation and to the Michaelis equation plus a term for nonsaturable migration as defined under “Experimental Procedures.” The resulting parameters for three experiments are shown in Table II. Although the sum of the squared residuals (the difference between the calculated and experimental values) is less when the nonsaturable term is included, it is not so much less that a choice in favor of the additional term can be made on this basis alone. The consistent value for V₉₅/K₉₅ obtained when the analysis includes the nonsaturable component provides additional evidence for its inclusion. When only the simple Michaelis equation is used the experimental rate values could include some component of transport which would not be accounted for by a rectangular hyperbola, and V₉₅/K₉₅ would therefore be too large. When the first-order component, Kₐ, is included in the descriptive equation to which the experimental rates are fitted, V₉₅/Kₐ becomes consistent, as would be expected for a saturated mediated component even when the contribution of the nonsaturable component varies among the experiments. The data for experiment 2 (Table II) and the calculated curves for the two hypothetical components are plotted in Fig. 3. The two different slopes found when the data are analyzed in an Eadie-Scatchard plot (Fig. 3, inset) also suggest two components, whether the second be saturable or not, within the experimental range.

We interpret these data to mean that in mouse L-929 lysosomes both a saturable and a nonsaturable pathway exist for passage of cystine. Because the influx for cystine is low in unloaded granules and presumably contains such a nonsaturable component, we have not been able to obtain data of sufficient precision to calculate reliable values for Kₐ and V₉₅, the parameters for the saturable component (zero-trans condition). The relationship between rate and external cystine concentration for these unloaded preparations is not significantly different from a straight line represented by a first-order rate constant of 3.5 nmol-unit of hexosaminidase⁻¹·min⁻¹·mm⁻¹. At 8 μM cystine, this rate would account for 28 pmol of cystine·unit of hexosaminidase⁻¹·min⁻¹ which approximates the highest rate for unloaded granules we have observed. Based on the Kₐ calculated from Table II for loaded granules, one could expect only 6–14 pmol of cystine·unit of hexosaminidase⁻¹·min⁻¹ to penetrate via the nonsaturable route. Possibly the net nonsaturable flux may be larger in the absence of cystine on the opposite side (zero-trans condition). In addition, although NEM has very little effect on countertransport at 8 μM external cystine, the effect of NEM, if any, on the nonsaturable component revealed at high external cystine concentrations has not yet been evaluated.

We emphasize that our failure to saturate completely the external cystine-binding site and our subsequent assignment of the flux remainder to a “nonsaturable” component do not exclude the possibility that such a component could, in fact, also be a saturable mediated system with a high Kₐ. We are limited experimentally to roughly 1.3 mM external cystine by its solubility, but we have attempted to circumvent this limitation by inundating the external binding site with a more soluble competitive inhibitor.

A Dixon plot (Fig. 4) shows that the effect of readily soluble L-selenomethionine is consistent with competitive inhibition because the family of curves crosses at a point above the y axis. The nonsaturable component contributes a significant portion of the uptake only at the highest external cystine concentration (see Fig. 3). By presentation of the data in the form of the Dixon plot shown in Fig. 4, the error in evaluation of the primary transporter is largely confined to those values in the plot for 408 μM external cystine. The value of Kₐ is derived from the slopes of the other curves and is represented in experiment 3 of Table II. The Kₐ for this experiment was derived from the difference between calculated and observed rates at 408 μM external cystine.

Having found a soluble competitive inhibitor, we applied
Lysosomal Cystine Transport

TABLE II

Kinetic parameters of cystine countertransport in mouse L-929 fibroblasts

| Cystine content | \( K_i \) | \( V_{\text{max}} \) | \( K_d \) | \( V_{\text{max}}/K_i \) |
|----------------|----------|----------------|--------|-----------------|
| nmol cystine  | mM       | nmol cystine  | mM     | nmol cystine    |
| unit hexosaminidase |       | unit hexosaminidase |       | unit hexosaminidase |

Fit to simple Michaelis equation only

Exp. 1
87.4 ± 0.60
0.60 ± 0.12
4.0 ± 0.83
7.5

Exp. 2
136
0.33 ± 0.04
0.49 ± 0.04
3.1 ± 0.18
6.4

Exp. 3
113
0.77 ± 0.09
2.0 ± 0.83
3.9 ± 0.29
5.1

Fit to Michaelis equation + nonsaturable

Exp. 1
87.4
0.33 ± 0.11
2.0 ± 0.83
1.8 ± 0.77
6.1

Exp. 2
136
0.28 ± 0.03
1.7 ± 0.21
0.8 ± 0.18
6.1

Exp. 3
113
0.21 ± 0.03
1.3
1.3
6.2

Fig. 3. Relation between rate of countertransport and exterior cystine concentration. Uptake of labeled cystine was measured as described under "Experimental Procedures" using increasing external cystine concentrations with a granular fraction preloaded to 136 nmol of cystine/unit of hexosaminidase. Observed values: the error bars represent standard error of the estimate obtained by linear regression of time points; ---, a plot from calculated values for Michaelis equation plus \( K_d \) \( [S] \) using parameters derived from nonlinear least squares fit to observed data; ..., a plot of saturable component generated by \( \mu_0 = 1.7 \) nmol of cystine-unit of hexosaminidase\(^{-1}\)·min\(^{-1}\), and \( K_s = 0.28 \) nmol of cystine-unit of hexosaminidase\(^{-1}\)·min\(^{-1}\)·mM\(^{-1}\) external cystine. Inset, ordinate \( v \) (nmol of cystine-unit of hexosaminidase\(^{-1}\)·min\(^{-1}\)·mM\(^{-1}\) external cystine); abscissa \( u \) (nmol of cystine-unit of hexosaminidase\(^{-1}\)·min\(^{-1}\)).

In the strategy of overwhelming the transporter with large concentrations of L-selenomethionine in an attempt to reach saturation of the primary transport system and expose a second if it should exist. Such a second system would not readily be inhibited within the test range because it is presumed to have an even larger \( K_i \). The results for L-selenomethionine and other competitors are shown in Fig. 5. The use of high concentrations of competitors does not imply that we believe that these competitors have any physiological role. Their use is a strategy to saturate the binding site with a soluble compound when it is impossible to do so with cystine. The extrapulation of these curves to maximum inhibition is charted in Table III, revealing an inhibitor-resistant component in some instances. Different values are found with different preparations as we have also observed in the estimates of \( K_s \) in Table II. Conceivably this alternative route varies in its contribution with the physiological state of the cells at the time of harvest. A similar variance in the nonsaturable component has also been reported for the anionic transport system (19). A study of the effect of temperature on cystine release has also led Forster et al. (20) to suggest an alternative pathway.

Inhibition by Cystine Analogs—We have used the inhibition of cystine countertransport by analogs of cystine, other amino acids, and their derivatives as a measure of recognition at the

Fig. 4. Inhibition of cystine countertransport by L-selenomethionine. The granular fraction was preloaded to 113 nmol of cystine/unit of hexosaminidase. Uptake of labeled cystine was measured as described under "Experimental Procedures" using the indicated external cystine concentrations with increasing external L-selenomethionine. Error bars represent the standard error of the estimate for each rate converted to fractional error of the reciprocal. Convergence of the curves indicates \( K_i = 12 \) mM and competitive inhibition.

Fig. 5. Inhibition of cystine countertransport in mouse L-929 fibroblasts. Countertransport was measured in the presence of inhibitors and expressed as percent of uninhibited control as described under "Experimental Procedures." Inhibitors and values for the initial uninhibited rates as pmol of cystine·min\(^{-1}\)·unit hexosaminidase were O, L-leucine, 62; X, L-selenomethionine, 43; X, L-cystathionine, 79; B, DL-selenocystine, 50; and A, L-cystine, 49.
binding site and by this means have explored, first of all, the structural requirements for that recognition by the lysosomal cystine transport system and second, whether cystine might utilize a previously described lysosomal amino acid transport system.

In order to be assured that comparisons among the various analog competition experiments are valid, we have included results only from preparations having a load level of 75 mmol of cystine/unit of β-hexosaminidase or more as the minimum value for inclusion. In these preparations the mean ± S.E. load level is 105 ± 5 (n = 27) mmol of cystine/unit of β-hexosaminidase (estimated 92% internal saturation), the mean ± S.E. uninhibited uptake rate at 8 μM external cystine is 50 ± 2 (n = 27) pmol of cystine·min⁻¹·unit hexosaminidase⁻¹. Using this guideline, the effect of the given analog expressed as percent of uninhibited control values is reproducible in different preparations on different days within a 10–15% standard error of the mean. Theoretically, when inhibition is expressed as percent of uninhibited control the value should be comparable for different preparations whether or not the preparations have the same internal saturation. Experimentally, however, there is a dependence on internal loading. We believe the reason for this is the presence of the second and variable transport component which is not subject to inhibition at the test concentrations. At low internal load levels the inhibitor-sensitive transport would comprise a smaller fraction of the total than it would at high load levels.

The results of the analog competition survey are presented in Tables IV and V. Some requirements for recognition by the primary cystine transporter and characteristics that differentiate the cystine transporter from other amino acid transport systems of lysosomes are indicated from these observations taken together. Table IV shows that the L-isomer is preferred by the recognition site of the mouse lysosomal cystine transporter, confirming and extending the observations of Gahl et al. (5) on the human leukocyte lysosomal cystine transporter. Whether both chiral centers of tetrapolar analogs must be of the L-configuration for recognition by the mouse system is uncertain. Several examples found in Tables IV and V considered together indicate that the presence of α-amino groups is more important than carboxylate groups for binding at the recognition site. LL-Lanthionine is a strong inhibitor; however, S-3,3-dipropionate, structurally equivalent to lanthionine but without its amino groups, is less effective. Although lacking carboxylate groups, the diamines above, inhibition might result from the competition of cystine monomethyl ester, derived by disulfide exchange, which either is not transported or which is transported carrying a depleted specific activity of label. We have shown, however, that the disulfide exchange measured by high voltage electrophoresis on paper amounts to only 1.1% of the total label under the conditions of the experiment. Most of the inhibition, then, must be the result of competition between CDME per se and cystine for the cystine transporter.

Table V also shows that sulfur seems to be accepted at the binding site as an apolar atom. The substitution of a sulfur atom for a carbon had little effect in the following paired examples: S-carboxymethyl-L-cysteine and L-2-aminoacidopente; thialysine and lysine; methionine and norleucine. A departure from this generalization is seen on comparing the inhibition of lanthionine and diaminopimelate, which also differ from each other structurally only in the presence of a single sulfur atom in place of carbon. Of this pair, the sulfur-containing analog is the better competitor (Table IV). These may be positioned more effectively by their four polar groups than analogs bearing a single α-amino and α-carboxylate but lacking either of the distal charged groups.

When selenium is substituted for sulfur in selenocysteine, selenocysteamine, and selenomethionine, the selenium-containing compound proves the better inhibitor. Although selenium may interact with proteins forming selenolsulfides, this atom may also be bound by hydrophobic interactions (21). In view of our collective experience with the binding of phenylglycine, methionine, leucine, isoleucine, and valine (Table IV), we believe that hydrophobic interactions may dominate at that part of the recognition site opposite the sulfur atoms of cystine. In addition to its greater mass compared with sulfur, selenium has approximately 10% larger effective diameter and slightly less electronegativity. The recognition site of the transporter accommodates selenium in place of sulfur so that the analog acts as a competitive inhibitor (Figs. 4 and 5).

Shape of the Apolar Domain—Examination of these molecular determinants for recognition of analogs by the binding site leads us to some rather more speculative deductions. If cystine should take a right- or left-handed spiral conformation with dihedral angles ranging from 80 to 103°, as do cystine residues found in crystalline proteins (22), or of 106°, as it does in crystalline cystine (23), then the central apolar domain of the cystine-binding site must provide sufficient space in three dimensions to accommodate such a shape. As illustrated in Fig. 6, left, the S-S bond may be visualized as the hinge of a door, and the dihedral angle formed by the flanking carbons may then be considered the angle to which the door is opened. If the C-S-S-C “door-hinge” of cystine were stretched open to 105°, the molecule would have the same angle as the C-S-C “door-hinge” in crystalline cystine (23), then the central apolar domain of the cystine-binding site must provide sufficient space in three dimensions to accommodate such a shape. As illustrated in Fig. 6, left, the S-S bond may be visualized as the hinge of a door, and the dihedral angle formed by the flanking carbons may then be considered the angle to which the door is opened. If the C-S-S-C “door-hinge” of cystine were stretched open to 105°, the molecule would have the same angle as the C-S-C “door-hinge” in crystalline cystine (23), then the central apolar domain of the cystine-binding site must provide sufficient space in three dimensions to accommodate such a shape. As illustrated in Fig. 6, left, the S-S bond may be visualized as the hinge of a door, and the dihedral angle formed by the flanking carbons may then be considered the angle to which the door is opened. If the C-S-S-C “door-hinge” of cystine were stretched open to 105°, the molecule would have the same angle as the C-S-C “door-hinge” in crystalline cystine (23), then the central apolar domain of the cystine-binding site must provide sufficient space in three dimensions to accommodate such a shape. As illustrated in Fig. 6, left, the S-S bond may be visualized as the hinge of a door, and the dihedral angle formed by the flanking carbons may then be considered the angle to which the door is opened.
### TABLE IV

**Effect of cystine analogs and dipolar amino acid competitors on cystine countertransport in granular fractions from mouse L-929 fibroblasts**

Rates for countertransport were determined as described under “Experimental Procedures.” Values separated by a semicolon were obtained on two different preparations. Values obtained on three or more different preparations are designated by the mean + S.E. (n). The mean ± S.E. (n) of uninhibited rate was 51 ± 12 (n = 25) pmol/min/unit of hexosaminidase, and the load level was 105 ± 5.6 (n = 25) nmol of cystine/unit of hexosaminidase for the preparations used for these experiments.

| Cystine analogs          | Countertransport % uninhibited | Dipolar amino acids (10 mM) | Countertransport % uninhibited |
|--------------------------|-------------------------------|-----------------------------|-------------------------------|
| 1 mM                     |                               |                             |                               |
| LL-Cystine               | 36; 39                        | L-Serine                    | 79                            |
| DD-Cystine               | 83                            | L-Threonine                 | 104                           |
| DL-Selenocystine         | 45                            | 2-Aminoisobutyrate          | 85                            |
| L(+)-Cystathionine       | 76                            | L-Alanine                   | 86                            |
| L(-)-allo-Cystathionine  | 81                            | L-Proline                   | 87                            |
| LL-Homocystine           | 89                            | L-Phenylglycine             | 45                            |
| LL-Djenkolate            | 101                           | L-Valine                    | 66                            |
|                          |                               | n-Valine                    | 91                            |
|                          |                               | 3-Methyl-L-valine           | 103                           |
| 10 mM                    |                               |                              |                               |
| L(+)-Cystathionine       | 33 ± 3 (n = 3)                | DL-Threonine sulfone        | 84                            |
| LL-Lanthionine           | 28                            | L-Methionine                | 51 ± 4 (n = 4)                |
| DD-Lanthionine           | 65                            | L-Methionine                | 90; 103                       |
| LL-Diaminopimelate       | 57 ± 8 (n = 3)                | L-Selenomethionine          | 42 ± 3 (n = 5)                |
| DD-Diaminopimelate       | 98                            | L-Methionine sulfoximine    | 84                            |
| meso-Diaminopimelate     | 115                           | L-Tryptophan                | 84                            |
| LL,DD-meso-4-exo-Methylenediaminopimelate | 109                         | L-Phenylalanine             | 78                            |
| 1,4-Diamino-dicarbocyclohexane | 72                         | BCH (40 mM)                 | 85                            |

### TABLE V

**Effect of analog charge on cystine countertransport in granular fractions from mouse L-929 fibroblasts**

See legend to Table IV. The mean ± S.E. (n) of the uninhibited rate was 52 ± 4.7 (n = 9) pmol/min/unit of hexosaminidase, and the load level was 102 ± 6.6 (n = 9) nmol of cystine/unit of hexosaminidase for the preparations used for these experiments.

| Compound                  | Concentration (mM) | Charge | Countertransport % uninhibited control |
|---------------------------|--------------------|--------|----------------------------------------|
| Cystamine                 | 10                 | ++     | 56                                     |
| Selenocystine             | 10                 | ++     | 30                                     |
| L-Cystine dimethyl ester  | 10                 | ++     | 47                                     |
| L-Ornithine               | 10                 | +      | 81                                     |
| L-Lysine                  | 10                 | +      | 69 ± 3 (n = 3)                         |
| N²-Methyl-L-lysine        | 10                 | +      | 71                                     |
| S-Ethylamino-L-cysteine   | 10                 | +      | 81; 86                                 |
| L-Arginine                | 10                 | +      | 79                                     |
| L-Homoarginine            | 10                 | +      | 82                                     |
| L-Histidine               | 10                 | 0      | 77                                     |
| N²-Acetyl-L-ornithine     | 10                 | 0      | 62                                     |
| L-Citrulline              | 10                 | 0      | 69                                     |
| L-Aspartate               | 10                 | −      | 73                                     |
| L-Glutamate               | 10                 | −      | 86                                     |
| S-Carboxyethyl-L-cysteine | 10                 | −      | 68                                     |
| S-Carboxymethyl-L-cysteine| 8.3                | −      | 69                                     |
| L-2-Aminoadipate          | 8.3                | −      | 51; 62                                 |
| S-3,3-Dipropionate        | 10                 | −      | 76                                     |

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*Net mean charge to nearest integral value; zwitterions are represented as 0 charge.

Labeled cystine-cysteamine disulfide was less than 1% of total label.

Labeled cystine monomethyl ester was 1.1% of total label.

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**FIG. 6.** Left, computer-generated diagram of left-hand spiral cystine as it might fit into the transporter recognition site. The dihedral angle is 90°. Right, computer-generated diagram of isoleucine on the same scale as it might fit into the recognition site of the cystine transporter.

The failure of homocystine (89%) or djenkolate (101%) to inhibit may indicate that the binding site is too short to accommodate these longer analogs of cystine.

**Relation to Other Amino Acid Transport Systems in Lysosomes**—Beyond a molecular description of the transporter site, the second goal of these studies is to determine whether cystine might be utilizing, in part, one of the previously described lysosomal amino acid carriers. The amino acids and analogs surveyed in Tables IV and V were chosen to provide a profile of inhibitors for this purpose. Those analogs shown in Table V demonstrate the effect of net charge and of substrates for lysosomal transport systems c (24) and d (19).

The presence or absence of a positive charge does not seem to increase the rather weak and variable inhibition by cationic amino acids. The cationic amino acid, N²-methyl-L-lysine, recognition site may be narrow in this region. The failure of homocystine (89%) or djenkolate (101%) to inhibit may indicate that the binding site is too short to accommodate these longer analogs of cystine.
which is accepted by the lysosomal transport system c, here shows no more competition than do citrulline and \(N^2\)-acetyl-L-ornithine, which lack a net charge. We conclude that cystine does not utilize transport system c, an observation confirmed by the reports that lysine does not exit the lysosome any faster when cystine is placed on the outside (25) and that cystinotic lysosomes ordinarily lose cystine only very slowly (8, 25) in spite of having a normal transport system c. Our observations also eliminate any significant contribution by the system of the epithelial membrane type for cystine which is characteristically shared with lysine and arginine (28).

The absence of significant effect by aspartate or glutamate excludes system d as a route for cystine flow in lysosomes. This is a conclusion that confirms the observation of Gahl et al. (5) that glutamate does not inhibit cystine countertransport in lysosomes of human leukocytes and of Collarini et al. (19) that cystine does not inhibit glutamate transport in human fibroblast lysosomes. Since glutamate does not stimulate transport either, we also confirm that plasma membrane cystine transport system \(\kappa_c\) is not detectable in lysosomes (27).

System e has been shown to carry alanine, serine, and threonine but not methionine; and systems f and p have been shown to carry proline (28). We see no indication that these pathways are utilized by cystine since methionine does inhibit, and the other four analogs have only minimal effect on cystine countertransport (Table IV). Tryptophan and phenylalanine, substrates for system t in human fibroblast lysosomes (29) or h in rat thyroid lysosomes (10), have little effect on the mouse lysosomal cystine transporter. Although methionine, together with branched chain amino acids, may be recognized both by the rat thyroid system h and the cystine transporter, cystine is not in turn recognized by system h (10). The specific cysteine transporter described by Pisoni et al. (30) would not carry even homocysteine, and therefore it would be implausible for it to carry cystine. Since our incubation mixtures contain NEM, cysteine that might egress from the lysosome would not exchange with the labeled external cystine and thus carry label into the lysosome as cysteine.

These observations exclude the identity of the cystine transporter with e, f, p, t, h, or the cystine transport systems. The effects of leucine, norleucine, and phenylglycine match rather well, with the exception of the ineffective RCH, the substrate pattern for lysosomal system 1 as described by Stewart et al. (29) in human fibroblasts. We are not able to show, however, significant trans-stimulation of leucine uptake by cystine loading (data not shown). This result would seem to eliminate system 1 for cystine recognition although the existence of a species difference must be considered. In the mouse experiments, for example, we found no large nonsaturable component for leucine transport when excess leucine was present, a characteristic that differs from the observations of Stewart et al. (29).

Other Known Cystine Transport Systems—In Escherichia coli there are two cystine transport systems that differ in sensitivity to inhibitors. The specific system recognizes and is therefore inhibited only by selenocystine and cystathionine in addition to cystine. The general system recognizes and is inhibited not only by cysteine, selenocystine, and cystathionine, but also by lanthionine, disminopimelate, 3-hydroxy- and 4-methylaminopimelate (31). A structural homology may conceivably be discovered between the recognition site of the general \(E. coli\) system and the mouse lysosomal cystine transporter. Lanthionine, which is a considerably more effective inhibitor of the general compared with the specific system in \(E. coli\), also reduces both mouse and human fibroblast lysosomal cystine countertransport to only 28% of control for 10 mM LL-lanthionine (data for human not shown). Furthermore, the mouse lysosomal transporter, like the general but not the specific \(E. coli\) system, shows a tolerance for branching at C-3 and C-4 (valine, leucine, isoleucine) and a modest inhibition by 10 mM L-(3R)-hydroxydiaminopimelate (62% of control). This similarity in the shape of the apolar domain between the primary mouse cystine transporter and the \(E. coli\) general system is another indication of possible homology.

Significance—The work in this report shows that lysosomal cystine probably cannot make significant use of the transport systems described previously for other amino acids. It may, however, follow to a small degree an alternative transmembrane route that we have designated provisionally as nonsaturable and which should not automatically be assigned to diffusion. Since cystine is generated from protein degradation within the lysosome and is reduced to cysteine on the cytoplasmic side of the lysosome immediately after exodus, it is evident that these pathways that we have studied by countertransport for convenience of manipulation must operate in the living cell primarily in the direction of exodus. The inability of lysosomes to utilize an adequate alternative route has led to the accumulation of cystine in persons lacking the primary cystine transporter, the defect of the inherited human disease, cystinosis. If the presence of a nonsaturable component, especially a variable one, is also demonstrated for human lysosomal cystine transport, it would provide an explanation for the observation that although cystinosis patients lack a functional cystine transporter, their cells do not accumulate cystine beyond a level that is typical for each individual and each cell type. It might, in addition, provide an explanation for the partial cystine transport ability of lysosomes observed in benign cystinosis (32). The characterization of this alternative route would improve the understanding of cystinosis and its variants.

The nature of the chemical and spatial determinants for binding to the recognition site of the recognized lysosomal cystine transporter may provide an understanding for the design of effective irreversible inhibitors which will mark the transport protein for eventual isolation and purification.

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REFERENCES

1. Schulman, J. D., Bradley, K. H., and Seegmiller, J. E. (1969) Science 166, 1152–1154
2. Gahl, W. A., Bashan, N., Tietze, F., Bernardini, I., and Schulman, J. D. (1982) Science 217, 1263–1265
3. Jonas, A. J., Smith, M. L., and Schneider, J. A. (1982) J. Biol. Chem. 257, 13185–13188
4. Jonas, A. J., Smith, M. L., Allison, W. S., Laikind, P. K., Greene, A. A., and Schneider, J. A. (1983) J. Biol. Chem. 258, 11727–11730
5. Gahl, W. A., Tietze, F., Rashan, N., Bernardini, I., Raiford, D., and Schulman, J. D. (1985) Biochem. J. 216, 393–400
6. Gahl, W. A., and Tietze, F. (1985) Biochem. J. 228, 263–267
7. Jonas, A. J. (1986) Biochem. J. 236, 671–677
8. Smith, M. L., Greene, A. A., Potashnik, R., Mendoza, S. A., and Schneider, J. A. (1987) J. Biol. Chem. 262, 1244–1253
9. Greene, A. A., and Schneider, J. A. (1987) Biochem. J. 246, 547–549
10. Bernar, J., Tietze, F., Kohn, L. D., Bernardini, I., Harper, G. S., Grollman, E. F., and Gahl, W. A. (1986) J. Biol. Chem. 261, 17107–17112

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11. Smith, M. L., Greene, A. A., Schneider, J. A., Pisoni, R. L., and Christensen, H. N. (1989) Methods Enzymol. 174, 154–162
12. Smith, M. L., Furlong, C. E., Greene, A. A., and Schneider, J. A. (1987) Methods Enzymol. 143, 144–148
13. Leroy, J. G., Ho, M. W., MacBrinn, M. C., Zielke, K., Jacob, J., and O’Brien, J. S. (1972) Pediatr. Res. 6, 752–757
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
15. Jonas, A. J., Speller, R. J., Conrad, P. B., and Dubinsky, W. P. (1989) J. Biol. Chem. 264, 4953–4956
16. Segel, I. H. (1975) in Enzyme Kinetics, pp 878–883, Wiley-Interscience, New York
17. White, M. F., and Christensen, H. N. (1982) J. Biol. Chem. 257, 10069–10080
18. Gahl, W. A., Bashan, N., Tietze, F., and Schulman, J. D. (1984) Am. J. Hum. Genet. 36, 277–282
19. Collarini, E. J., Pisoni, R. L., and Christensen, H. N. (1989) Biochim. Biophys. Acta 987, 139–144
20. Forster, S., Scarlett, L., and Lloyd, J. B. (1989) Biochim. Biophys. Acta 1013, 7–10
21. Ganther, H. E., and Kraus, R. J. (1987) Methods Enzymol. 143, 32–38
22. Katz, B. A., and Kossiakoff, A. (1986) J. Biol. Chem. 261, 15480–15485
23. Oughton, B. M., and Harrison, P. M. (1959) Acta Crystallogr. 12, 396–404
24. Pisoni, R. L., Thoene, J. G., Lemone, R. M., and Christensen, H. N. (1987) J. Biol. Chem. 262, 15011–15018
25. Pisoni, R. L., Thoene, J. G., and Christensen, H. N. (1985) J. Biol. Chem. 260, 4781–4789
26. Dent, C. E., and Rose, G. A. (1951) Q. J. Med. 20, 205–219
27. Christensen, H. N. (1988) Biosci. Rep. 8, 121–129
28. Pisoni, R. L., Flickinger, K. S., Thoene, J. G., and Christensen, H. N. (1987) J. Biol. Chem. 262, 6010–6017
29. Stewart, B. H., Collarini, E., J., Pisoni, R., and Christensen, H. N. (1989) Biochim. Biophys. Acta 987, 145–153
30. Pisoni, R. L., Acker, T. L., Lisowski, K. M., Lemons, R. M., and Thoene, J. G. (1990) J. Cell Biol. 110, 327–335
31. Berger, E. A., and Heppel, L. A. (1972) J. Biol. Chem. 247, 7684–7693
32. Gahl, W. A., and Tietze, F. (1987) Pediatr. Res. 21, 103–106
33. Inui, Y., and Christensen, H. N. (1966) J. Gen. Physiol. 50, 203–224
Characterization of the lysosomal cystine transport system in mouse L-929 fibroblasts.
A A Greene, E G Marcusson, G P Morell and J A Schneider

J. Biol. Chem. 1990, 265:9888-9895.

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