Tyrosine countertransport was used to demonstrate the existence of a carrier system for neutral amino acids in the lysosomal membrane of FRTL-5 thyroid cells. In addition to tyrosine, the carrier system recognized the neutral amino acids leucine, histidine, phenylalanine, and tryptophan. Cystine and lysine, amino acids for which a lysosomal carrier system has been demonstrated, showed no competition with tyrosine for countertransport. The tyrosine system showed stereospecificity and cation independence. It did not require an acidic lysosome or the availability of free thiols. The apparent $K_a$ for tyrosine was approximately 100 $\mu$M; the energy of activation of the system was approximately 9.7 kcal/mol. This new lysosomal membrane carrier system for neutral amino acids resembles the plasma membrane L system in 3T3 Chinese hamster ovary cells and melanoma B-16 cells.

Several systems for the transport of small molecules across the lysosomal membrane have been described. Carrier-mediated transport of the amino acid cystine was demonstrated in 1982; a deficiency of this transport system was shown to be responsible for the autosomal recessive disease nephropathic cystinosis (1-4). Later, a lysosomal transport system which recognized lysine, other cationic amino acids, and the mixed disulfide cysteine-cysteamine was reported (5). Recently, lysosomal egress of the monosaccharide, sialic acid, was shown (7, 17). The very high levels of intralysosomal tyrosine achieved by this technique have permitted the demonstration of tyrosine countertransport. This phenomenon, in which tracer amounts of a radiolabeled substrate will cross a membrane at an increased rate if there is a substantial concentration of the nonradioactive substance on the opposite side of the membrane, rejects the concept of a simple pore and constitutes strong evidence for a carrier-mediated process (10).

Lysosomal tyrosine transport was studied in human polymorphonuclear leukocytes and in a rat thyroid-derived cell line, FRTL-5. A carrier-mediated system for lysosomal transport of tyrosine and other neutral amino acids was demonstrated in the rat cells.

MATERIALS AND METHODS

Radiolabeled L-tyrosine methyl ester was prepared by methylation of radioactive L-tyrosine (Amersham Corp., 50 Ci/mmol) in 3 N anhydrous methanolic HCl (2). Nonradioactive amino acid methyl esters were purchased from Sigma. Human polymorphonuclear leukocytes were isolated from whole blood by dextran sedimentation and hypotonic lysis of erythrocytes (2).

FRTL-5 cells, a continuous line of functional epithelial cells from rat thyroid which require the presence of thyroid-stimulating hormone for growth (11, 12), were grown in monolayer culture in 10-cm Petri dishes at 37°C in a humidified atmosphere of 5% CO$_2$:95% air. Coon's modified Ham's F-12 medium (NIH media unit) was supplemented with 5% heat-inactivated calf serum (GIBCO), 1 mM nonessential amino acid (Microbiological Associates, Bethesda, MD), thyroid-stimulating hormone (1 x 10$^{-9}$ M), insulin (10 $\mu$g/ml), cortisol (10$^{-5}$ M), transferrin (5 $\mu$g/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), somatostatin (10 ng/ml), and glucose (6.0%). The medium was changed every 3-4 days, and cells were passaged on reaching confluency using a trypsin/collagenase chick serum mixture (11, 12).

Preparation of Tyrosine-loaded Granular Fractions—Intact human polymorphonuclear leukocyes were exposed to 0.08-0.6 mM [3H] tyrosine methyl ester (final specific radioactivity 18 Ci/mmol) in Hanks' balanced salt solution containing 10 mM sodium phosphate, pH 7, to achieve various tyrosine loadings. Cells were ruptured by limited sonication using a model W 140 cell disruptor with a microtip (Head Systems, Ultrasonic, Inc., Plainview, NY), and a lysosome-rich granular fraction, loaded with tyrosine, was prepared by differential centrifugation as previously described (2, 4).

Intact rat thyroid FRTL-5 cells were exposed to [3H]tyrosine methyl ester for egress experiments, or nonradioactive tyrosine methyl ester for countertransport, in Hanks' balanced salt solution containing 10 mM sodium phosphate. Cells were centrifuged, resuspended in 0.25 M sucrose, 10 mM Hepes, pH 7.0, and subjected to limited sonication. The homogenate was centrifuged at 2,000 g for 5 min and the supernatant centrifuged at 17,000 $\times$ g for 10 min to prepare a crude granular fraction pellet. This pellet contained approximately 30% of the total hexosaminidase activity in the homogenate and was 1.5-2.5-fold enriched with respect to this lysosomal enzyme. Electron microscopy of the crude granular fraction obtained after fixing in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (13), followed by post-fixation in 1% osmium tetroxide, staining with 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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aqueous uranyl acetate, and embedding with propylene oxide/Spurr resin mixture (14), revealed many single membrane vesicular structures characteristic of lysosomes, as well as numerous mitochondria and abundant cell debris (Fig. 1).

Tyrosine Egress—Egress experiments were performed on human leucocytes as well as rat thyroid cells. Crude granular fractions, loaded with $[^{3}H]$tyrosine, were suspended in sucrose/Hepes and incubated at $37^\circ C$. Aliquots were removed at various times and centrifuged at $17,000 \times g$ for 10 min, and radioactivity was measured in supernatant and pellet. High-voltage electrophoresis (2) verified that the radioactivity represented only free tyrosine and not tyrosine methyl ester. Tyrosine present in the supernatant due to rupture of lysosomes during incubation was corrected for by measurement of soluble hexosaminidase (1).

Tyrosine Countertransport—Tyrosine-loaded and -unloaded granular fractions from FRTL-5 cells were suspended in sucrose/Hepes containing 8 $\mu M$ $[^{3}H]$tyrosine and incubated at $37^\circ C$. At various times, aliquots were removed and centrifuged at $17,000 \times g$ for 10 min at $4^\circ C$. The supernatant was assayed for soluble hexosaminidase activity, whose mean value was 5.6% of total hexosaminidase at zero time and 6.6% after 3 min at $37^\circ C$. The pellet was washed vigorously with 1 ml of sucrose/Hepes, once by resuspension with a Pasteur pipette, and once by resuspension in a glass pestle, with subsequent centrifugation (4). Final recovery of hexosaminidase in the pellet averaged 74% of initial activity. The radioactivity in duplicate 100-$\mu l$ aliquots was determined using a Beckman scintillation counter. $[^{3}H]$Tyrosine uptake was expressed as counts/min/unit of hexosaminidase at the designated time minus the zero-time value. The difference between the uptake into the loaded lysosomes and uptake into the unloaded lysosomes constituted countertransport and was expressed as picomoles/unit of hexosaminidase/min. Within a single experiment, duplicate determinations differed by 7–10%. Radioactivity was converted to picomoles of tyrosine by dividing by the specific activity of $[^{3}H]$tyrosine in the incubation medium.

To differentiate binding from true uptake into lysosomes, tyrosine-loaded granular fractions, obtained at the end of a typical countertransport experiment and containing large amounts of $[^{3}H]$tyrosine, were placed on 2.4-cm GF/A glass fiber filters (Whatman) and washed extensively with sucrose/Hepes. Another aliquot was ruptured by sonication and treated similarly. The tyrosine-loaded granular fractions lost 70% of their radioactivity after sonication. This amounted to 12 times the radioactivity lost from unloaded granular fractions due to sonication, indicating that the bulk of $[^{3}H]$tyrosine uptake into loaded granular fractions was released by lysosomal rupture and represented true uptake rather than enhanced binding.

To assess lysosomal loading with nonradioactive tyrosine, a 100-$\mu l$ aliquot of the crude granular fraction was assayed for tyrosine using an LKB 4150 amino acid analyzer with a 5-buffer lithium citrate system.

Assay of Hexosaminidase Activity—The extent of lysosomal rupture was assessed by dividing supernatant hexosaminidase activity by total hexosaminidase activity in the granular fraction suspension. Hexosaminidase activity was assayed by 3 min incubation in 13 mM citric acid, 20 mM sodium phosphate, pH 4.4, as previously described (1). One unit of hexosaminidase hydrolyzed 1 nmol of substrate/min at $37^\circ C$.

Protein was measured by the method of Lowry et al. (15).
RESULTS

Loading of lysosomes with tyrosine was accomplished by exposure of intact cells to tyrosine methyl ester. For whole FRTL-5 cells, tyrosine loading was maximal at pH 7.2 (Fig. 2a) and increased with duration of tyrosine methyl ester exposure up to 20 min (Fig. 2b). Therefore, a pH of 7.2 and an exposure time of 20 min were chosen for all subsequent loadings. Under these conditions, granular fraction tyrosine loading increased linearly with tyrosine methyl ester concentrations up to 3.0 mM (data not shown).

For human leucocytes and rat FRTL-5 cells, the crude granular fractions obtained from cells exposed to 0.6–1.0 mM tyrosine methyl ester contained 10–20 times the amount of free tyrosine compared with granular fractions from cells not exposed to the methyl ester. This high level of tyrosine loading permitted determination of a rate of tyrosine egress out of granular fractions for each cell type (Fig. 3). Tyrosine exited leucocyte granular fractions very slowly but left the FRTL-5 cell granular fractions at a high rate. In view of this finding, and because we could not demonstrate tyrosine countertransport in the leucocyte granular fractions (data not shown), subsequent experiments were performed using the FRTL-5 cells.

Tyrosine countertransport was investigated in these cells by measuring granular fraction uptake at 8 μM [3H]tyrosine. L-Tyrosine-loaded granular fractions took up 5 times more [3H]tyrosine than did unloaded granular fractions (Fig. 4); the difference comprised tyrosine countertransport. To eliminate the possibility that the difference in [3H]tyrosine content represented nonspecific uptake, it was demonstrated that cystine-loaded granular fractions took up no more [3H]tyrosine than did unloaded granular fractions (Table I).

Since tyrosine countertransport increased linearly with time through at least 3 min (Fig. 4), an initial velocity of [3H]tyrosine uptake, in picomoles of tyrosine/unit of hexosaminidase/min, could be measured after 3 min of incubation. This initial velocity increased with increased loading of nonradioactive tyrosine up to approximately 1.5 nmol of tyrosine/unit of hexosaminidase and then leveled off (Fig. 5). This demonstration of saturation kinetics confirmed that tyrosine was being transported by a carrier-mediated system. To ensure maximal [3H]tyrosine uptake in subsequent experiments, the granular fractions were routinely loaded to at least 1.5 nmol of tyrosine/unit of hexosaminidase; this level was generally achieved by exposing the whole cells to 1–2 mM tyrosine methyl ester.

The velocity of tyrosine countertransport increased as temperature increased (Fig. 6), with a Q10 of approximately 1.9. The energy of activation was 9.7 kcal/mol.

The technique of tyrosine countertransport was also used to determine which compounds compete with [3H]tyrosine for uptake into tyrosine-loaded granular fractions; such com-
TABLE I

| Intralysosomal amino acid | [3H]Cystine uptake | [3H]Tyrosine uptake pmol/hexosaminidase unit |
|---------------------------|-------------------|---------------------------------------------|
| None                      | 0                 | 0.31                                        |
| Cystine                   | 5.7               | 0.29                                        |
| Tyrosine                  | 0.03              | 0.62                                        |

DISCUSSION

Carrier-mediated transport of tyrosine and other neutral amino acids across the plasma membrane has been described for 3T3 Chinese hamster ovary cells (16) and melanoma B-16 cells (17). The system has an apparent $K_m$ for tyrosine of 75 µM and is sodium-independent (16, 17). It was characterized by measuring uptake of radiolabeled amino acids into whole cells.
TABLE II

| Compound | Concentration | Transport |
|----------|---------------|-----------|
| L-Tyrosine | 8 | 100 |
| | 80 | 63 |
| | 100 | 47 |
| | 160 | 36 |
| | 500 | 8 |
| D-Tyrosine | 500 | 71 |
| Group I | | |
| L-Cystine | 500 | 95 |
| L-Lysine | 500 | 101 |
| L-Glycine | 500 | 118 |
| L-Glutamate | 500 | 91 |
| L-Proline | 500 | 105 |
| L-Alanine | 500 | 87 |
| Group II | | |
| L-Valine | 500 | 45 |
| L-Methionine | 500 | 23 |
| Group III | | |
| L-Isoleucine | 500 | 18 |
| L-Histidine | 500 | 16 |
| | 100 | 47 |
| L-Phenylalanine | 500 | 3 |
| | 100 | 24 |
| L-Leucine | 500 | 5 |
| | 100 | 45 |

TABLE III

| Compound present | [3H]Tyrosine uptake | % of control |
|------------------|---------------------|-------------|
| None             | 100                 | 100         |
| NH4Cl (20 mM)    | 100                 | 87          |
| N-Ethylmaleimide (5 mM) | 100 | 87 |
| NaCl (100 mM)    | 100                 | 90          |
| KCl (100 mM)     | 100                 | 90          |
| MgCl2 (2 mM)     | 104                 | 90          |
| MgATP (2 mM)     | 90                  | 92          |
| NaATP (2 mM)     | 92                  |             |

Effects of ammonium chloride, N-ethylmaleimide, various cations, and ATP on tyrosine countertransport in FRTL-5 granular fractions

FRTL-5 granular fractions were loaded to over 2.1 nmol of tyrosine/unit of hexosaminidase. [3H]Tyrosine uptake was measured in the presence of the added compounds and expressed as a percentage of control uptake, which averaged 1.85 ± 0.07 pmol of [3H]tyrosine uptake/unit of hexosaminidase/min.

Such uptake experiments are more difficult with subcellular vesicles such as lysosomes, although this technique has recently been used by Pisoni et al. (18) to elucidate a transport system for proline and other neutral amino acids in Percoll-purified fibroblast lysosomes. An alternative method for studying lysosomal membrane transport is to load the lysosomes with a substance and measure its egress from the vesicles (1, 2, 5, 6, 9) or its stimulation of uptake into the vesicles (4). Exposure of cells to an amino acid methyl ester has been shown specifically to load lysosomes with the free amino acid (8); the amino acid esterase activity copurifies with lysosomes, and lysosomal accumulation of the free amino acid is inhibited by the lysosomotropic alkalinizing agent, chloroquine (9). The crude granular fractions which we studied after whole cell exposure to tyrosine methyl ester contained electron-dense bodies typical of lysosomes and no other organelles known to have hydrolytic activity against amino acid methyl esters (Fig. 1). Furthermore, high-voltage electrophoretic analysis verified that the tyrosine content of granular fractions from methyl ester-treated cells was increased 10–20-fold compared with granular fractions from untreated cells.

It has been shown that lysosomes could be loaded with each of the neutral amino acids leucine (9), phenylalanine (19), and tyrosine by exposure to the respective amino acid methyl ester. However, Reeves (9) was unable to demonstrate a trans effect of external leucine on leucine exodus in rat liver lysosomes, Pisoni et al. (5) could not detect a trans effect by neutral amino acids on phenylalanine exodus from human fibroblast lysosomes, and we were unable to show a trans effect on egress of tyrosine using human leukocyte lysosomes; furthermore, we could not demonstrate countertransport of tyrosine in the same system (data not shown). These findings suggest that, for reasons perhaps related to the saturability of the lysosomal carrier or to species variability, the rat liver and human cells studied were not appropriate for the demonstration of a neutral amino acid carrier in the lysosomal membrane.

In contrast, lysosome-rich granular fractions from rat FRTL-5 cells provided a suitable system for the study of lysosomal tyrosine transport (Fig. 3). They were appropriately loaded by exposure to tyrosine methyl ester (Fig. 2) and displayed several characteristics of carrier-mediated transport. The first and the most important criterion of facilitated transport was the demonstration of countertransport itself; [3H]tyrosine uptake was greater in tyrosine-loaded compared with unloaded granular fractions (Fig. 4). This was true uptake, rather than some nonspecific binding enhanced by prior exposure to tyrosine methyl ester, because bound [3H]tyrosine (determined by radioactivity retained on a glass fiber filter after extensive sonication) was the same for loaded and unloaded fractions exposed to [3H]tyrosine. The countertransport was also specific for tyrosine, compared with another amino acid, cystine, transported by a lysosomal membrane system (1–4). That is, cystine loading did not enhance [3H]tyrosine uptake into granular fractions, and tyrosine loading did not enhance [3H]cystine uptake (Table I). Second, the tyrosine transport system was stereoselective for the L-isomer. L-Tyrosine at 0.5 mM competed with [3H]tyrosine uptake only negligibly compared with L-tyrosine (Table II). Third, tyrosine countertransport appeared a saturable process with respect to intralysosomal tyrosine loading. Finally, the Qo for tyrosine transport (1.9) was in a range consistent with carrier-mediated transport rather than simple diffusion; the Qo closely resembled that for lysosomal cystine transport (2.0) in human leucocytes (4).

In the rat FRTL-5 cells, the lysosomal carrier for tyrosine also recognized other neutral amino acids, including leucine, phenylalanine, tryptophan, histidine, and isoleucine (Table II). The relatively broad specificity for ligands resembled that reported for the lysine (5) and proline (18) carriers in fibroblast lysosomes, but contrasted with the strict ligand requirements of the human leucocyte lysosomal cystine transport system (4). The ligand affinity for the tyrosine and cystine systems also differed. Although the cystine carrier had an estimated K, for cystine of 0.5 mM (4), approximately 0.1 mM tyrosine competed 50% with [3H]tyrosine for uptake (Table II). However, as for the cystine system, lysosomal tyrosine transport was not influenced by alkalinization with amo-
Lysosomal Tyrosine Transport in Rat Thyroid Cells

Countertransport has been employed to show the gene-dosage effect of a deficiency of the lysosomal cystine carrier (25), implying that the technique may permit a determination of the abundance of a given carrier within the lysosomal membrane. If so, then manipulating the intracellular metabolism of growing rat thyroid cells, with subsequent assay of the number of lysosomal tyrosine carriers, could reveal important characteristics about the biochemistry of lysosomal membrane carrier proteins.

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The rat cell system for lysosomal tyrosine transport resembles the plasma membrane L system in ligand specificity (Table II), approximate K_0 for tyrosine binding (17) (Table II), and sodium independence (Table III). These similarities, along with the fact that the lysosomal system does not depend upon intravesicular acidification (Table III), prompt speculation that the same gene might code for this particular neutral amino acid carrier in the plasma membrane and in the lysosomal membrane, with conservation among different species. The implication that an amino acid transport protein can be targeted to either the plasma membrane or the lysosomal membrane has been suggested by Pisoni et al. (5), based upon the resemblance of the lysosomal lysine carrier system and the plasma membrane system (21-23). This targeting hypothesis may be tested using the tyrosine-neutral amino acid systems by employing a photofaffinity probe, such as p-azidophenylalanine as suggested by Tabb et al. (24), to purify both the L system carrier from plasma membranes (16, 17) and the tyrosine carrier from rat lysosomal membranes. These investigations would comprise the first steps in comparing the plasma and lysosomal membrane carriers and elucidating the normal pathway for incorporation of carrier proteins into their respective membranes.

The transport system for tyrosine reported here is the most recent lysosomal membrane carrier system to be described, following those for cystine (1-4), lysine (5), sialic acid (6), and proline (18). However, it is the only lysosomal system in which countertransport is demonstrable in growing cells.