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Characteristics of cystine counter-transport in normal and cystinotic lysosome-rich leucocyte granular fractions

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1. Normal leucocyte lysosome-rich granular fractions exhibited counter-transport of cystine, confirming that cystine transport across the lysosomal membrane is carrier-mediated. 2. The trans-activation of cystine transport was temperature-dependent but relatively independent of the external Na^+ or K^+ concentration in phosphate buffer. 3. Counter-transport, measured as uptake of exogenous [^{3}H]cystine, increased with increasing intralysosomal cystine content up to approx. 3 nmol of half-cystine/unit of hexosaminidase activity. 4. The amount of [^{3}H]cystine entering lysosomes loaded with unlabelled cystine decreased when unlabelled cystine was added to the extralysosomal medium. 5. Lysosomal cystine counter-transport was stereospecific for the L-isomer. Cystathionine, cystamine and cysteamine–cysteine mixed disulphide gave evidence of sharing the lysosomal cystine-transport system, although at lower activity than cystine. Other tested amino acids, including arginine, glutamate and homocystine, were inactive in this system. 6. Nine leucocyte lysosome-rich preparations from eight different cystinotic patients displayed virtually no counter-transport of cystine, conclusively establishing that a carrier-mediated system for cystine transport is dysfunctional in cystinotic lysosomes.

Exposure of rat liver (Goldman & Kaplan, 1973; Reeves, 1979) or human leucocyte (Steinherz *et al.*, 1982*a*) granular fractions to certain amino acid methyl esters results in intralysosomal ester hydrolysis and accumulation of the corresponding free amino acid. This method was used to measure cystine egress from intact human leucocytes (Steinherz *et al.*, 1982*b*) and from isolated lysosomal-rich granular fractions (Gahl *et al.*, 1982*a*). The exodus of cystine from the isolated lysosomal preparations was matched by the appearance of cystine in the supernatant after the granular fractions had been centrifuged (Gahl *et al.*, 1982*a*). In normal leucocyte lysosomes, the velocity of cystine egress was unaltered by 1 mM-*N*-ethylmaleimide and reached a maximum rate as cystine loading of the lysosomes increased, suggesting a saturable transport system (Gahl *et al.*, 1982*b*).

The same studies were performed in leucocytes from patients with cystinosis, a lysosomal storage disease in which free cystine accumulates within lysosomes of various cells (Schneider & Schulman, 1982; Schulman, 1973). There was negligible egress of cystine from whole leucocytes (Steinherz *et al.*, 1982*b*) or from isolated cystinotic lysosomes (Gahl *et al.*, 1982*a*), suggesting that a cystine-transport system normally present in lysosomal membranes is defective in cystinosis.

The above studies were of special interest because previously there was no experimental support for the concept that lysosomal amino acid transport might, in some cases, be carrier-mediated. Indeed, it

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appeared that egress of certain amino acids other than cystine from lysosomes might be diffusional (Reeves, 1979; Steinherz *et al.*, 1982*a*). Furthermore, our own studies of lysosomal cystine egress, which demonstrated saturation kinetics (Gahl *et al.*, 1982*b*), were of necessity accomplished at lysosomal cystine loadings that probably approximate the physical solubility of this compound at intralysosomal pH. Therefore we attempted to determine whether lysosomal cystine transport is accompanied by counter-transport, which constitutes classical evidence for a carrier-mediated (as opposed to diffusional) system of transmembrane movement (Wilbrandt & Rosenberg, 1961). In counter-transport, tracer amounts of a radiolabelled substance will cross a membrane at an increased rate if there is a substantial concentration of the non-radioactive substance on the opposite side of the membrane.

We now report that normal lysosomal cystine transport exhibits trans-activation and that there is little or no counter-transport of cystine across cystinotic lysosomal membranes. We also describe the properties of normal counter-transport of L-cystine with respect to saturability, temperature, cation requirements, stereospecificity and competition with cystine analogues and various amino acids.

Experimental

L-[^{3}H]Cystine (3200, 1100 or 876 mCi/mmol, L-[^{3}H]methionine (75 Ci/mmol), L-[^{3}H]phenylalanine (58 Ci/mmol). [^{35}S]homocystine (18.8 mCi/mmol) and L-[^{35}S]cystathionine (11.8 mCi/mmol) were obtained from Amersham International. [^{3}H]Arginine (15 Ci/mmol), L-[^{3}H]leucine (158 Ci/mmol), L-[^{3}H]tryptophan (8 Ci/mmol), L-[^{3}H]tyrosine (34.7 Ci/mmol), L-[^{14}C]alanine (153 mCi/mmol), L-[^{14}C]glutamate (297 mCi/mmol) and L-[^{35}S]cystine (456 Ci/mmol) were products of New England Nuclear.

The mixed disulphides of L-cysteine with D-penicillamine and β-thiopropionic acid were prepared by reaction of L-cystine disulphoxide (Emiliozzi & Pichat, 1959) with the appropriate thiols under the conditions described by Eriksson & Eriksson (1967) for the preparation of cysteine–glutathione mixed disulphide. The filtered reaction mixtures were applied to the top of a Dowex 1 (formate form) column, and the components were separated with a formic acid gradient (Eriksson & Eriksson, 1967). Fractions containing the desired components, identified by high-voltage paper electrophoresis, were pooled, concentrated to small volumes and treated with excess acetone to precipitate the mixed disulphides. For preparation of L-cysteine–cysteamine mixed disulphide the products of the reaction of cystine disulphoxide with cysteamine (mercaptoethylamine) were separated on a column of Bio-Rex 70 (Na^+ form) (Bio-Rad Laboratories), with 1 mM-formic acid as eluent. The final product, though essentially free of cysteinesulphinic acid and cystamine, contained 6–10% cystine, as determined by analyses on a Beckman model 121M amino acid analyser.

Human polymorphonuclear-leucocyte-rich white blood cells, 0.05–0.10 ml packed volume in 4 ml of Hanks balanced salt solution (GIBCO), were exposed to unlabelled cystine dimethyl ester, usually 1 mM, for 30 min at 37°C, as previously described (Steinherz *et al.*, 1982*b*; Gahl *et al.*, 1982*a*). Control cells were not exposed to the methyl ester. After washing and sonication of the cells at 4°C in 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid]/NaOH buffer, pH 7.0, containing 0.25 M-sucrose, a post-nuclear granular fraction (Gahl *et al.*, 1982*a*) was prepared by centrifugation at 8500 ***g*** for 10 min. This was resuspended in cold 0.25 M-sucrose containing 1 mM-*N*-ethylmaleimide (Pierce Chemical Co.) and either 10 mM-Hepes/NaOH buffer, pH 7.0, or 10 mM-sodium phosphate buffer, pH 6.0, and incubated at 37°C in the presence of 8 μM-[^{3}H]cystine. At zero and later times, samples were removed and centrifuged at 17000 ***g*** for 10 min. The supernatant was assayed for soluble hexosaminidase activity, which averaged approx. 8% of total hexosaminidase at zero time and approx. 16% of total after 60 min at 37°C. The pellet was washed twice with 2 ml of buffered sucrose, once by resuspension with a Pasteur pipette and once by resuspension with a glass pestle, with subsequent centrifugation. Final hexosaminidase recovery in the pellet approximated 63% of initial activity. The final pellet was taken up in 1 ml of buffered sucrose plus 10 μl of 4% (v/v) Triton X-100. The radioactivities of duplicate 100 μl portions were counted in a Beckman scintillation counter and assayed for hexosaminidase activity with 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (1.2 mM) (Koch–Light Laboratories) in citrate buffer as previously described (Gahl *et al.*, 1982*b*). [^{3}H]Cystine entrance was expressed as c.p.m./unit of hexosaminidase at the designated time minus the zero-time value. The radioactivity value was converted into pmol of cystine by dividing by the c.p.m./pmol of the [^{3}H]cystine in the incubation medium (Fig. 5). High-voltage electrophoresis was employed to verify that [^{3}H]cystine had entered as cystine (Steinherz *et al.*, 1982*a*; Gahl *et al.*, 1982*a*) and that [^{35}S]-cystathionine had entered as cystathionine (Table 1). To assess loading with unlabelled cystine, the cystine-binding-protein assay method of Oshima *et al.* (1974) was used.

Results

A normal leucocyte granular fraction was loaded with unlabelled cystine to concentrations saturating with respect to cystine egress (Gahl *et al.*, 1982*b*) and then exposed to tracer quantities of radioactive cystine. [^{3}H]Cystine entrance increased approximately linearly with time of incubation until at least 60 min and plateaued by approx. 120 min at 37°C (Fig. 1). The ^{3}H label that entered was identified as [^{3}H]cystine by high-voltage electrophoresis of granular-fraction acid extracts (Steinherz *et al.*, 1982*a*; Gahl *et al.*, 1982*b*). Uptake was reversible: if a cystine-loaded granular fraction was exposed to [^{3}H]cystine for 2 h, washed thoroughly at 4°C, resuspended in sucrose/Hepes buffer, pH 7.0, containing 1 mM-*N*-ethylmaleimide, and then kept at 37°C for 1 h, radioactivity re-accumulated outside the lysosomes at a substantial rate not accounted for by lysosomal rupture (results not shown). Granular fractions not loaded with cystine showed virtually no [^{3}H]cystine uptake (Fig. 1).

In control experiments, granular fractions from leucocytes exposed to 0.25 mM-tryptophan methyl ester took up negligible [^{3}H], as did cystine-loaded

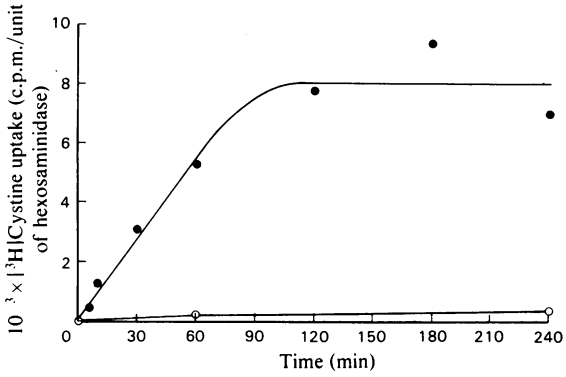

Fig. 1. *Time course of* [^{3}H]*cystine entrance into normal leucocyte granular fractions loaded or not loaded with unlabelled cystine*

Human leucocytes were exposed to either no (○) or 1 mM (●) unlabelled cystine dimethyl ester, and granular fractions were placed in 0.25 M-sucrose/1 mM-*N*-ethylmaleimide/10 mM-Hepes/NaOH buffer, pH 7.0, containing 7.2 μM-[^{3}H]cystine (1.1 Ci/mmol) at 37°C. Samples of granular fraction were removed at different times, washed, assayed for total hexosaminidase activity and their radioactivities counted as described in the text.

Table 1. *Uptake of radioactive compounds by normal leucocyte granular fractions loaded with unlabelled cystine*

Normal leucocytes were exposed to no or 1 mM-cystine dimethyl ester for 30 min at 37°C. Granular fractions were prepared and placed in 0.25 M-sucrose/1 mM-*N*-ethylmaleimide in either 10 mM-sodium phosphate buffer, pH 6.0 (Expt. I) or 10 mM-Hepes/NaOH buffer, pH 7.0 (Expts. II and III), plus the radioactive compound diluted with unlabelled chemical to achieve the listed concentration. Entrance of label after 1 h at 37°C was determined as described in the Experimental section and the value was converted into pmol by dividing the compound's specific radioactivity. Mean soluble hexosaminidase activity at zero time (6.7% for non-cystine-loaded lysosomes; 10.0% for cystine-loaded lysosomes) and at 1 h (13.3% for non-cystine-loaded lysosomes; 17.8% for cystine-loaded lysosomes) was not increased in the presence of any of the added compounds. Expt. I: loaded granular fractions contained 6.4 nmol of half-cystine/unit of hexosaminidase activity. All ^{3}H-labelled compounds were adjusted to 876 mCi/mmol; [^{14}C]alanine specific radioactivity was 87.6 mCi/mmol; [^{14}C]glutamate specific radioactivity was 280 mCi/mmol. Expt. II: loaded granular fractions contained 4.4 nmol of half-cystine/unit of hexosaminidase activity. The ^{3}H-labelled compounds were adjusted to a specific radioactivity of 876 mCi/mmol. Expt. III: loaded granular fractions contained 6.5 nmol of half-cystine/unit of hexosaminidase activity. [^{35}S]Cystine, [^{35}S]homocystine and [^{35}S]cystathionine were adjusted to approx. 6.5, 13.1 and 8.3 mCi/mmol respectively.

		Lysosomal uptake (pmol/unit of hexosaminidase per h)			Lysosomal uptake ratio (loaded/not loaded)
	Extralysosomal compound	Not loaded	Loaded	Difference	
Expt. I	L-[^{3}H]Cystine (8 μM)	0.18	17.98	17.8	99.9
	L-[^{3}H]Methionine (8 μM)	4.15	3.17	0	0.8
	L-[^{3}H]Tryptophan (8 μM)	1.39	4.25	2.9	3.1
	L-[^{3}H]Tyrosine (8 μM)	1.43	3.21	1.8	2.2
	L-[^{3}H]Leucine (8 μM)	0.91	1.85	0.9	2.0
	L-[^{3}H]Phenylalanine (8 μM)	0.13	1.48	1.4	11.4
	L-[^{14}C]Alanine (8 μM)	0	0	0	—
	L-[^{14}C]Glutamate (8 μM)	0.16	0.26	0.1	1.6
Expt II	L-[^{3}H]Cystine (8 μM)	0.17	11.82	11.7	69.5
	L-[^{3}H]Tryptophan (8 μM)	2.56	5.50	2.9	2.1
	L-[^{3}H]Tyrosine (8 μM)	1.34	2.97	1.6	2.2
	L-[^{3}H]Arginine (8 μM)	1.98	4.61	2.6	2.3
Expt. III	L-[^{35}S]Cystine (408 μM)	20.4	484.5	465.1	23.8
	L-[^{35}S]Homocystine (408 μM)	65.8	67.5	1.7	1.0
	L-[^{35}S]Cystathionine (408 μM)	21.5	141.0	119.5	6.6

granular fractions that had been freeze–thawed thrice (results not shown). Moreover, cystine-loaded granular fractions exposed to radioactive amino acids other than cystine did not take up substantial amounts of label (Table 1).

For cystine-loaded lysosomes exposed to [^{3}H]-cystine for 1h, uptake of radioactivity/unit of hexosaminidase was constant over a range of 0.6–13.7 units of hexosaminidase per 1 ml sample. In most experiments reported in the present paper the samples contained lysosomes with 1–5 units of hexosaminidase activity. Background or non-specific binding of [^{3}H]cystine to granular fractions was diminished considerably by including 1 mM-*N*-ethylmaleimide in the external medium.

Cystine counter-transport was temperature-dependent (Fig. 2), with a Q_{10} of approx. 2.0. The estimated energy of activation was 47.7 kJ/mol (11.4 kcal/mol).

Normal cystine counter-transport was similar in phosphate buffer at pH 6.0 and Hepes buffer at pH 7.0. Subsequent experiments were performed in 0.25 M-sucrose/1 mM-*N*-ethylmaleimide in either 10 mM-sodium phosphate buffer, pH 6.0, or 10 mM-Hepes/NaOH buffer, pH 7.0. [^{3}H]Cystine counter-transport was relatively independent of the KCl or NaCl concentration in the phosphate buffer (Fig. 3).

The rate of [^{3}H]cystine uptake increased with increasing amounts of intralysosomal cystine loading, then tended to level off (Fig. 4). Because the greatest rate of counter-transport was achieved at loadings above 3 nmol of half-cystine/unit of hexosaminidase, such loadings were used to generate the data in all the other Figures.

As the final concentration of extralysosomal L-cystine, with tracer [^{3}H]cystine, was progressively increased, total cystine uptake by the loaded normal granular fractions increased with a gradually decreasing slope (Fig. 5). Supporting data are also found in Table 2. The highest extralysosomal L-cystine concentration that could be tested was limited by the solubility of cystine at pH 6.0. A crude estimate of maximum uptake of L-cystine would be approx. 600 pmol of half-cystine/unit of hexosaminidase per h, not very different from the maximum observed exodus rate of 15 pmol of

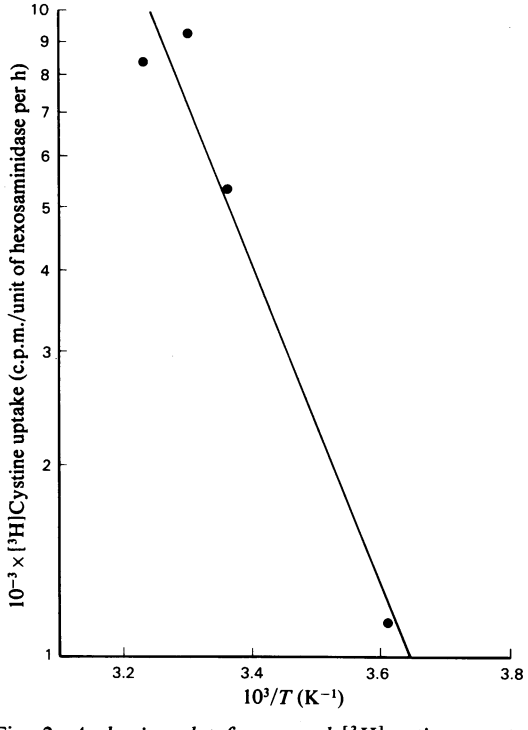

Fig. 2. *Arrhenius plot for normal [^{3}H]cystine counter-transport by leucocyte granular fractions*
Cystine-loaded granular fractions, loaded to 3.7 nmol of half-cystine/unit of hexosaminidase, were placed in 0.25 M-sucrose/1 mM-*N*-ethylmaleimide/10 mM-Hepes/NaOH buffer, pH 7.0, containing 7.3 μM-[^{3}H]cystine at 4, 25, 30 or 37°C. Uptake after 60 min, per unit of hexosaminidase, was plotted as a logarithmic function of reciprocal temperature in degrees Kelvin.

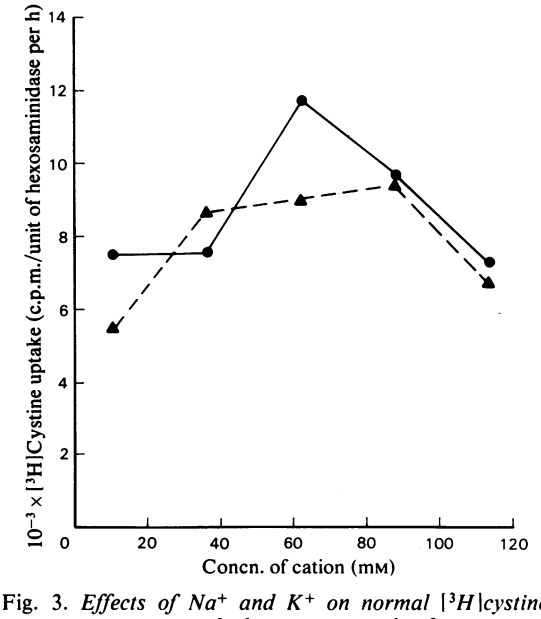

Fig. 3. *Effects of Na^+ and K^+ on normal [^{3}H]cystine counter-transport by leucocyte granular fractions*
Leucocyte granular fractions were loaded to 5.4 nmol of half-cystine/unit of hexosaminidase and incubated in medium buffered with 10 mM-sodium phosphate, pH 6.0, containing 7.5 μM-[^{3}H]cystine (876 mCi/mmol) and 10–113 mM-NaCl (●) or -KCl (▲). Final molarity was maintained at 300 mM by using sucrose.

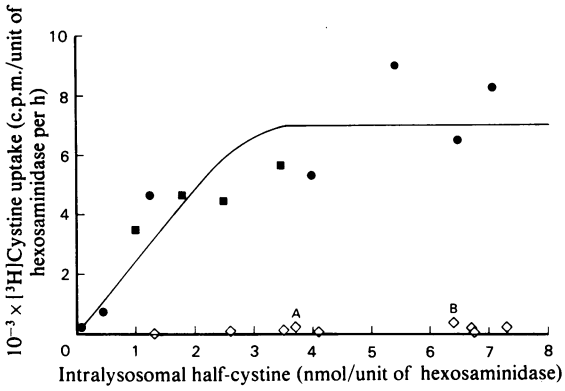

Fig. 4. [^{3}H]*Cystine uptake by normal and cystinotic leucocyte granular fractions loaded to different concentrations with unlabelled cystine*

Normal leucocytes were exposed to 0–2.0mM-cystine dimethyl ester, and their respective granular fractions were incubated at 37°C in 0.25M-sucrose/1mM-*N*-ethylmaleimide/10mM-sodium phosphate buffer, pH6.0, containing 7.5μM-[^{3}H]cystine (876mCi/mmol). Uptake of radioactivity per hexosaminidase unit was determined after 1h. ● and ■ symbols represent different experiments. Cystinotic leucocytes from eight different patients (◊) were exposed to 1mM-cystine dimethyl ester and their granular fractions were incubated at 37°C for 1h in 0.25M-sucrose/1mM-*N*-ethylmaleimide containing 5.2–7.6μM-[^{3}H]cystine (876–1100mCi/mmol) in 10mM-Hepes/NaOH buffer, pH7.0, 25mM-sodium phosphate buffer, pH6.0, or 10mM-sodium phosphate buffer, pH6.0. Most cystinotic leucocytes were obtained 5h after an oral dose of cysteamine (approx. 15mg/kg); patient A was receiving cysteamine at a lower dosage and patient B had received no cysteamine for 24h.

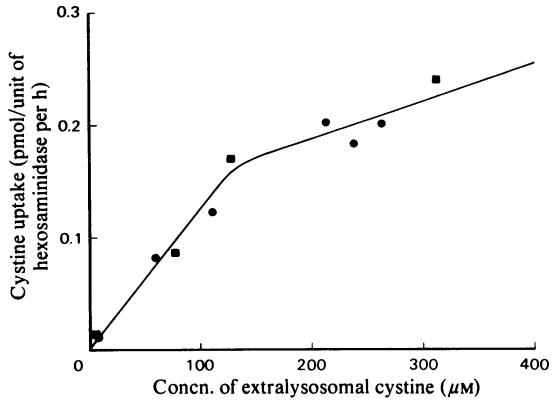

Fig. 5. *Leucocyte granular-fraction counter-transport in the presence of different concentrations of* L-*cystine*

In separate experiments, normal leucocytes were loaded with 1mM-cystine dimethyl ester to 6.8nmol (■) and 4.5nmol (●) of half-cystine/unit of hexosaminidase. Samples were added to 0.25M-sucrose/1mM-*N*-ethylmaleimide/10mM-sodium phosphate buffer, pH6.0, containing 6.7μM-[^{3}H]cystine (876mCi/mmol) plus different amounts of unlabelled L-cystine, whose concentration was confirmed by the assay with cystine-binding protein. Portions were removed at 0 and 60min, washed, their radioactivities counted, and assayed for hexosaminidase. Values for radioactivity (c.p.m.) inside the lysosomes were converted into pmol of entering cystine by using the calculated specific radioactivity of the cystine in the incubation buffer. [^{3}H]Cystine uptake increased more slowly as the extralysosomal L-cystine concentration exceeded approx. 150μM, suggesting competition between radioactive and non-radioactive cystine for a limited number of transport carrier molecules.

half-cystine/unit of hexosaminidase per min previously reported (Gahl *et al.*, 1982*b*). (The calculation of pmol of L-cystine taken up by the lysosomes represents a minimum estimate, since dilution of the extralysosomal [^{3}H]cystine by exiting intralysosomal unlabelled L-cystine was not considered.)

The technique of cystine counter-transport was used to determine which compounds share the cystine-transport system with cystine. Two approaches were employed: non-radioactive compounds were tested for competition with tracer [^{3}H]cystine for entrance into cystine-loaded lysosomes (Table 2), and radioactive compounds were examined for direct entrance into cystine-loaded lysosomes (Table 1). In the former studies, it was first shown that non-radioactive extralysosomal cystine at 0.08–0.86mM did decrease the amount of tracer [^{3}H]cystine entering normal cystine-loaded lysosomes (Table 2). In fact, an approximate K_m of 0.5mM can be calculated for the cystine-transport system. D-Cystine at 0.86mM, as well as at lower concentrations, did not compete with [^{3}H]cystine for counter-transport. Nor was their substantial competition with any of several 4.4mM-L-amino acids, including the dibasic amino acid arginine, or with 1.8mM-L-homocystine. L-Cystathionine and *S*-ethylcysteine, at 1.8mM, decreased entrance of radioactive cystine by 31% and 18% respectively, and DL-homocysteic acid, β-carboxyethylthiocysteine, *S*-methylcysteine, the *N*-ethylmaleimide adduct of cysteine and D-penicillamine–L-cysteine disulphide did not substantially affect [^{3}H]cystine counter-transport. The D-penicillamine disulphide at 1mM caused excessive lysosomal rupture and could not be tested. At 1.6mM cystamine decreased [^{3}H]cystine entrance by 56%; this could not be attributed to contamination with cystine, which was measured at less than 0.05% of the cystamine concentration. Cysteamine–cysteine disulphide, at approx. 1.8mM, inhibited cystine counter-transport by 45%; measured contamination of this disulphide with cystine (0.1–

Table 2. *Competition with [^{3}H]cystine for counter-transport into normal cystine-loaded leucocyte lysosomes*

In four representative experiments, normal leucocytes were exposed to 1 mM-cystine dimethyl ester to achieve lysosomal loading of over 4 nmol of half-cystine/unit of hexosaminidase activity. Granular fractions were placed in 0.25 M-sucrose/10 mM-Hepes containing 1 mM-*N*-ethylmaleimide, 6.8–7.4 μM-[^{3}H]cystine and the added compound, and the pH was adjusted to 6.9–7.1. Soluble hexosaminidase was no greater for lysosomes exposed to any tested compound than for those with no added compound (control). Mean control [^{3}H]-cystine counter-transport for the four experiments was 16.8 (range 14.1–18.2) pmol of [^{3}H]cystine/unit of hexosaminidase per h at 37°C.

Added compound	Transport (% of control)
—	100
L-Cystine* (0.08 mM)	82
(0.16 mM)	79
(0.37 mM)	45
(0.45 mM)	35
(0.63 mM)	43
(0.82 mM)	18
(0.86 mM)	25
D-Cystine† (0.86 mM)	111
L-Arginine (4.4 mM)	102
L-Methionine (4.4 mM)	88
L-Alanine (4.4 mM)	86
L-Tryptophan (4.4 mM)	98
L-Tyrosine (4.4 mM)	101
L-Glutamate (4.4 mM)	95
L-Homocystine (0.9 mM)	88
(1.8 mM)	90
L(+)-Cystathionine (0.9 mM)	85
(1.8 mM)	69
DL-Homocysteic acid (1.8 mM)	96
β-Carboxyethyl-L-thiocysteine (1.8 mM)	93
S-Methylcysteine (1.8 mM)	101
S-Ethylcysteine (0.9 mM)	101
(1.8 mM)	82
N-Ethylmaleimide–L-cysteine adduct (1.8 mM)	97
D-Penicillamine–L-cysteine mixed disulphide (1.8 mM)	89
Cysteamine–L-cysteine mixed disulphide (1.6–1.9 mM‡)	55
Cystamine§ (1.6 mM)	44

* Concentration determined by assay with cystine-binding protein.

† A saturated solution was prepared in parallel with saturated L-cystine, whose concentration was measured by the assay with cystine-binding protein. Other preparations of D-cystine also did not compete with [^{3}H]cystine for entrance.

‡ Determined by amino acid analyser; contained 6–10% (molar ratio) of cystine.

§ Contained less than 0.01% of L-cystine by assay with cystine-binding protein and less than 0.05% of cystine by amino acid analyser.

0.2 mM) could account for only approx. 20% inhibition of cystine counter-transport.

The second method of assessing what molecules share the cystine-carrier system involved measuring the entrance of radioactive compounds into cystine-loaded compared with non-cystine-loaded normal lysosomes. For 8 μM-[^{3}H]cystine, the difference between entrance into cystine-loaded and non-cystine-loaded lysosomes, which constitutes counter-transport, was 17.8 (Expt. I) or 11.7 (Expt. II) pmol/unit of hexosaminidase per h (Table 1). [^{3}H]Tryptophan and [^{3}H]arginine at 8 μM showed a greater entrance, by 2.9 and 2.6 pmol/unit of hexosaminidase per h, into cystine-loaded compared with non-cystine-loaded lysosomes, with a smaller effect for [^{3}H]tyrosine. However, the fold difference, i.e. ratio of entrance (cystine-loaded/not-loaded), was 70–100 for [^{3}H]cystine uptake, but only 2–3% of this for most of the other ^{3}H-labelled amino acids listed. [^{3}H]Phenylalanine exhibited approx. 10% of the counter-transport activity of [^{3}H]cystine when gauged by either the difference or the fold methods.

[^{35}S]Cystine, [^{35}S]homocystine and L-[^{35}S]cystathionine were each adjusted to 408 μM outside cystine-loaded or non-cystine-loaded granular fractions, and entrance of ^{35}S-labelled compound was measured. The higher concentration of cystine gave rise to greater entry of cystine due to counter-transport (465.1 pmol/unit of hexosaminidase per h), although the fold increase over entry into non-loaded lysosomes was somewhat decreased (23.8) compared with 8 μM-cystine. [^{35}S]Homocystine showed insignificant cystine-loading-dependent entry into normal lysosomes, whereas [^{35}S]cystathionine at 408 μM exhibited approx. 25% of the counter-transport that 408 μM-cystine displayed; for cystathionine this represented an approx. 7-fold stimulation by preloading the lysosomes with cystine. ^{35}S label in this latter experiment was demonstrated to remain in cystathionine by high-voltage electrophoresis (Steinherz *et al.*, 1982*a*; Gahl *et al.*, 1982*a*).

Nine cystinotic lysosome preparations from eight different patients were loaded to different concentrations with non-radioactive cystine. Each took up negligible [^{3}H]cystine after 1 h at 37°C (Fig. 5). Patient's blood samples were obtained 5 h after a dose of cysteamine, a cystine-depleting agent, except for patient B, who had not received cysteamine for 24 h. We have previously demonstrated that cysteamine administration *in vivo* does not modify the rate of cystine egress from cystinotic leucocytes (Steinherz *et al.*, 1982*b*) or their isolated lysosomes (Gahl *et al.*, 1982*a*,*b*).

Discussion

The demonstration of a lysosomal transport system for cystine provides new insights as to the

mechanisms by which certain small molecules, produced in part through enzymic catabolism of macromolecules, may escape from the intralysosomal space. The first for which evidence indicates a lysosomal carrier, cystine, has M_r 240, which may be expected to impair it from crossing the lysosomal membrane at an appreciable rate by simple diffusion (Schulman, 1973; Goldman, 1973).

Cystine egress from normal and cystinotic human lysosomes could be studied by our modification of a loading technique developed by Goldman & Kaplan (1973) and by Reeves (1979) for the study of rat liver lysosomes. Goldman & Kaplan (1973) found that the methyl esters of certain amino acids were hydrolysed inside isolated lysosomes to yield high concentrations of free amino acids. Reeves (1979) exploited this phenomenon to study the egress of certain radioactive amino acids (but not cystine) from rat liver lysosomes, and showed that non-lysosomal contamination did not interfere with the results. In our laboratory, this technique was extended to isolated human leucocyte granular fractions, and cystine was shown to be among the amino acids for which loading of lysosomes could be achieved by the methyl ester method (Steinherz *et al.*, 1982*a*). We subsequently observed that the methyl ester method could be used to load normal and cystinotic lysosomes within whole leucocytes with cystine (Steinherz *et al.*, 1982*b*), and that there was a measurable rate of cystine egress from normal, but not from cystinotic, human lysosomes isolated from such cystine-loaded leucocytes (Gahl *et al.*, 1982*a*). Supporting evidence has been reported by others (Jonas *et al.*, 1982). When velocity of normal cystine egress was plotted against the initial intralysosomal cystine loading, the saturability of the normal lysosomal cystine-transport system was suggested (Gahl *et al.*, 1982*b*).

Demonstration of saturability provided evidence that normal lysosomal cystine transport was carrier-mediated. However, the utility of any 'egress' system for studying transport was limited by the restricted ability to alter and measure conditions inside the intact lysosomes. For example, we know of no way to load normal lysosomes simultaneously with predictable concentrations of both cystine and another compound, which would be required to test such compounds in an egress system for competition with cystine via a putative carrier. In addition, the egress system measures cystine movement only down its concentration gradient, and studies carrier binding only under conditions of cystine excess, and unbinding in dilute (external) cystine solutions.

The counter-transport system, on the other hand, permits precise modification of the extralysosomal environment, and detailed study of various aspects of cystine transport. For example, counter-transport measurement has allowed straightforward demonstration of what compounds compete with cystine for its carrier. Furthermore, in contrast with egress, counter-transport is not substantially influenced by unlabelled cystine leaking from loaded lysosomes, because at the low extralysosomal [^{3}H]cystine concentration used entrance is a linear function of extralysosomal cystine concentration and dilution with unlabelled cystine does not alter the total radioactivity taken up. Counter-transport also effectively measures cystine transport at very dilute cystine concentrations.

The counter-transport systems shows unambiguously that cystine movement across normal lysosomal membranes is carrier-mediated (Fig. 1), of roughly equal magnitude in both directions (Gahl *et al.*, 1982*b*) (Fig. 5 and the Results section), and defective in cystinosis (Gahl *et al.*, 1982*a*,*b*) (Fig. 4). [In addition, the counter-transport technique may assist in diagnosing heterozygotes for cystinosis (W. A. Gahl, N. Bashan, F. Tietze & J. D. Schulman, unpublished work).] It has revealed a minimal dependence of cystine transport on external Na^+ or K^+ concentration in phosphate buffer (Fig. 3). It has also shown that the carrier is stereospecific for L-cystine and does not accept certain other amino acids for transport (Table 1), notably arginine, which shares with cystine a renal tubular-cell and intestinal-cell plasma-membrane transport system in humans (Dent & Rose, 1951), nor L-glutamate, whose transport into human fibroblasts is shared with cystine (Bannai & Kitamura, 1980). On the other hand, cystathionine, cystamine and the cysteamine–cysteine disulphide each gave evidence of competition with or actual transport by the cystine carrier (Tables 1 and 2). It should be noted that L-cystine was a far better competitor against [^{3}H]cystine uptake than was any other compound tested, and that the sharing of the cystine-transport system by the cysteamine–cysteine disulphide in no way eliminates the possibility that the mixed disulphide may also exit from lysosomes by another means, e.g. simple diffusion. This concept is central to a working hypothesis on the mechanism of cystine depletion by cysteamine in cystinosis (Thoene *et al.*, 1976).

Lysosomes provide various degradatory functions. Some of the resulting products are molecules that may not readily diffuse out of the lysosomes, and for these compounds lysosomal membrane transport systems may normally exist. For example, Docherty *et al.* (1979), using the osmotic-protection method, offered evidence for carrier-mediated sugar transport across rat liver lysosome membranes. Therefore the cystine carrier is likely to be only the first of such transport systems to be identified in man. The elucidation of other transport systems may be aided by studying counter-transport of small molecules in the normal state and in diseases with

lysosomal storage of small-M_r compounds. One may speculate, for example, that certain forms of free-sialic acid-storage diseases (Horwitz *et al.*, 1981) or certain defects in vitamin-B_{12} metabolism causing combined methylmalonicacidaemia and homocystinaemia (Mudd & Levy, 1978) could result from defective lysosomal transmembrane transport.

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