Impaired clearance of free cystine from lysosome-enriched granular fractions of I-cell-disease fibroblasts

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Cultured fibroblasts from patients with I-cell disease (mucopolipidosis II) accumulate excessive amounts of free cystine, similarly to cells from patients with nephropathic cystinosis, a disorder of lysosomal cystine transport. To clarify whether the intralysosomal accumulation of cystine in I-cell-disease fibroblasts was due to a defective disposal mechanism, we measured the rates of clearance of free [³⁵S]cystine from intact normal, cystinotic and I-cell-disease fibroblasts. Loss of radioactivity from the two mutant cell types occurred slowly ($t_{1/2}$ = 500 min) compared with the rapid loss from normal cells ($t_{1/2}$ = 40 min). Lysosome-rich granular fractions isolated from three different cystine-loaded normal, cystinotic and I-cell-disease fibroblast strains were similarly examined for non-radioactive cystine egress. Normal granular fractions lost cystine rapidly (mean $t_{1/2}$ = 43 min), whereas cystinotic granular fractions did not lose any cystine (mean $t_{1/2}$ = ∞). I-cell-disease granular fractions displayed prolonged half-times for cystine disposal (mean = 108 min), suggesting that I-cell-disease fibroblasts, like cystinotic cells, possess a defective carrier mechanism for cystine transport.

INTRODUCTION

Cultured fibroblasts from I-cell-disease (mucopolipidosis II) patients are severely deficient in UDP-*N*-acetylglucosamine-lysosomal-enzyme *N*-acetylglucosaminophosphotransferase (EC 2.7.8.17), a Golgi enzyme required for the formation of 6-phosphomannosyl residues on the carbohydrate portion of many lysosomal enzymes (Reitman & Kornfeld, 1981a,b; Hasilik *et al.*, 1981). Since these residues function as recognition markers for the incorporation of glycoproteins within lysosomes (Kaplan *et al.*, 1977; Sly & Fischer, 1982), I-cell-disease fibroblasts and other cells demonstrate a virtual absence of several acid hydrolases and a massive intralysosomal accumulation of partially hydrolysed cellular polymers (Neufeld & McKusick, 1983). We have previously reported (Tietze & Butler, 1979) that I-cell-disease fibroblasts also store large amounts of free (non-peptide) cystine, similarly to fibroblasts from patients with nephropathic cystinosis, another inherited lysosomal storage disorder (Schneider & Schulman, 1983). As indicated previously (Tietze, 1976; Jonas *et al.*, 1981) and shown here in fuller detail, cystine storage in I-cell-disease fibroblasts is localized primarily in the lysosome-rich granular compartment. Since lysosomal cystine transport has been characterized as carrier-mediated (Gahl *et al.*, 1982a, 1983; Gahl & Tietze, 1985) and defective in cystinotic leucocytes (Gahl *et al.*, 1982a,b, 1983, 1984a; Jonas *et al.*, 1982), the accumulation of free cystine in I-cell-disease lysosomes might imply a defective disposal mechanism. Alternatively, it may merely reflect a higher steady-state concentration of cystine within the greatly increased fractional volume of lysosomes which are present in I-cell-disease fibroblasts (Hanai *et al.*, 1971; Hollemans *et al.*, 1981). In order to resolve this issue, we compared the rates of disposal of free cystine from normal, cystinotic and I-cell-disease

fibroblasts, as well as from their isolated granular fractions. The results of this investigation provide evidence for defective clearance of free cystine from I-cell-disease fibroblast lysosomes. A preliminary account of portions of this work has appeared (Gahl *et al.*, 1984b).

EXPERIMENTAL

Materials

L-Cystine and its dimethyl ester were obtained from Sigma. The NEM adducts of L-cystine and GSH were prepared as previously described (Tietze & Butler, 1979). L-[³⁵S]Cystine dimethyl ester was prepared as a 1 mCi/ml solution in methanol from L-[³⁵S]cystine (Amersham; 176 mCi/mmol) as described by Steinherz *et al.* (1982b). Phosphate-buffered saline (154 mM-NaCl / 5 mM-NaH₂PO₄/1.5 mM-KH₂PO₄, pH 7.2) was obtained from Biofluids Inc., Rockville, MD, U.S.A.

Cell culture

Fibroblast strains designated by the prefix *GM* were purchased from the Human Genetic Mutant Cell Repository, Camden, NJ, U.S.A. Normal strains *Sc* and *Fa*, as well as I-cell-disease strains *Pf*, *Fy*, *Pe* and *He*, were obtained through the kindness of Dr. E. F. Neufeld; all other strains were grown from skin-punch biopsies of patients with nephropathic cystinosis or from frozen stocks maintained in these laboratories. Cells were cultured in 75 cm² or 150 cm² Falcon flasks in Eagle's minimal essential medium containing 10% (v/v) fetal-calf serum and non-essential amino acids (complete medium).

[³⁵S]Cystine clearance from whole fibroblasts

Normal cells were loaded with [³⁵S]-cystine by incubation of the confluent cell layer with 10 ml of serum-

Abbreviation used: NEM, *N*-ethylmaleimide.

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and cystine-free Eagle's medium containing 0.25–0.50 mM-L-[³⁵S]cystine dimethyl ester (0.58–1.0 μ Ci/ml) at 37 °C for 60 min. The highly penetrant ester diffuses into the lysosomal compartment and is hydrolysed to free cystine by esterase action (Goldman & Kaplan, 1973; Reeves, 1979). Cystinotic and I-cell-disease fibroblasts with elevated endogenous contents of free cystine were labelled by incubation for 48 h at 37 °C in medium containing 1–2 μ Ci of [³⁵S]cystine/ml of high specific radioactivity (367 Ci/mmol; New England Nuclear). Duplicate cultures of the cells, not exposed to label, were incubated at the same time and assayed for free cystine at the time of initiation of clearance; these assays established the contents of free cystine in the mutant cells at zero time of clearance and gave a measure of [³⁵S]cystine specific radioactivity.

After the [³⁵S]cystine loading, the cell layers were washed with phosphate-buffered saline and incubated at 37 °C with serum- and cystine-free (Expt. 1) or complete (Expt. 2) Eagle's medium. Flasks were withdrawn at 0, 30, 60 and 120 min, washed three times with phosphate-buffered saline, and treated with trypsin (0.25%; 10 ml; GIBCO) at 37 °C. The detached cells were washed three times at 0–4 °C by suspension and centrifugation in 5 ml of phosphate-buffered saline, and the cell pellet was sonicated in 0.5 ml of 10 mM-NEM in 10 mM-sodium phosphate buffer, pH 7.0, and treated with 50 μ l of 40% (w/v) sulphosalicylic acid. After 30 min on ice, the suspension was centrifuged (1000 *g*, 5 min). The supernatant was withdrawn and analysed by high-voltage paper electrophoresis for ³⁵S-containing substances, with un-

labelled cystine, cystine dimethyl ester, cysteine-NEM and GSH-NEM as markers (Steinherz *et al.*, 1982b). The pellet was dissolved in warm 0.5 M-NaOH and assayed for protein.

Cystine egress from isolated granular fractions

In four experiments, three different strains each of normal, cystinotic and I-cell-disease fibroblasts were harvested by trypsin treatment at confluency. Normal cell suspensions were loaded with non-radioactive cystine by exposure to 0.5 mM-cystine dimethyl ester for 30 min at 37 °C. All cells were washed with 5 ml of 0.25 M-sucrose and lysed by nitrogen cavitation [30 lb/in² (210 kPa), 10 min] (Rome *et al.*, 1979). The 1000 *g* (5 min) supernatant was centrifuged at 17000 *g* for 10 min to prepare a granular fraction, which was resuspended in 4.0 ml of 0.4 M-sucrose/16 mM-Hepes, pH 7.0, containing 1 mM-disodium EDTA, 1% bovine serum albumin and 1 mM-NEM. After 0–50 min at 37 °C, samples were removed for measurements of enzyme activities (hexosaminidase and acid phosphatase); 500 μ l portions were centrifuged at 17000 *g* for 10 min, and supernatants and pellets (resuspended in water) were assayed for cystine and for enzymic activities.

Lysosomal rupture, gauged by the ratio of supernatant to total lysosomal enzyme activity at each time point, averaged 19% at zero time and 29% after 50 min; the increase in soluble enzymic activity did not differ significantly among normal, cystinotic and I-cell-disease groups.

Cystine was assayed by the cystine-binding-protein

Table 1. Distribution of free cystine and acid hydrolase activities in subcellular fractions of cystinotic and I-cell-disease fibroblasts

Cells were ruptured by nitrogen cavitation in 0.25 M-sucrose, and fractions from differential centrifugations were analysed for cystine and α -mannosidase activity as described in the Experimental section. Fractions: Nuc, nuclear; Lys, lysosomal; Mit, mitochondria; Mic, microsomal; Supt, 100000 *g* supernatant fraction.

Expt. no.	Cell line	Genotype	Fraction	α -Mannosidase		Half-cystine	
				(units)	(%)	(nmol)	(%)
1	Wi	Cystinotic	Nuc	478	23	51.5	33
			Mit-Lys	1378	66	101	64
			Mic	48	2	1.5	1
			Supt	185	9	3.6	2
	He	I-cell	Nuc	77	17	1.66	17
			Mit-Lys	310	70	7.25	75
			Mic	25	6	0.30	3
			Supt	34	8	0.52	5
2	Pa	Cystinotic	Nuc	98	3	2.75	10
			Mit-Lys	2158	77	20.9	77
			Mic	119	4	0.65	2
			Supt	436	16	2.69	10
	GM 1586	I-cell	Nuc	24	4	25.5	16
			Mit-Lys	372	64	106	68
			Mic	67	12	1.68	1
			Supt	115	20	23.8	15
3	Fy	I-cell	Nuc	125	20	0.82	13
			Mit-Lys	347	56	3.30	53
			Mic	51	6	0.14	2
			Supt	98	16	1.91	31
	Pe	I-cell	Nuc	56	13	10.6	45
			Mit-Lys	269	63	11.6	49
			Mic	42	10	1.37	6
			Supt	59	14	0.25	1

Fig. 1. Fractionation of post-nuclear supernatants from cystinotic fibroblasts (a) and from I-cell fibroblasts (b) on colloidal-silica gradients

Eluate fractions were analysed for lysosomal α -mannosidase and for trichloroacetic acid-soluble ^{35}S -labelled metabolites as described in the Experimental section. Increasing density is from right to left. Legend: —, α -mannosidase activity; ----, $[^{35}\text{S}]$ cystine; ····, $[^{35}\text{S}]$ GSH.

method of Oshima *et al.* (1974). Cystine clearance was calculated by plotting the natural log of cystine content per unit of enzyme or protein against time (min) (Gahl *et al.*, 1982b). Velocity of cystine egress was calculated as described by Gahl *et al.* (1982a), by using both soluble hexosaminidase and acid phosphatase activities as measures of lysosomal rupture. The assays for hexosaminidase activity (Gahl *et al.*, 1982b), α -mannosidase activity (Rome *et al.*, 1979) and acid phosphatase activity (Hall *et al.*, 1978) have been previously reported; the use of the last two assays was of particular advantage in these studies, because of the relatively high amounts of these enzymes in the acid-hydrolase-deficient I-cell-disease fibroblasts (Leroy *et al.*, 1972; Wenger *et al.*, 1976). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Subcellular fractionation of cystinotic and I-cell-disease fibroblasts

These fibroblasts were washed, harvested by trypsin treatment and disrupted by low-pressure nitrogen cavitation as described above. The post-nuclear supernatant fraction, obtained by centrifugation of the homogenate at 1000 *g* for 5 min, was further sedimented at 17000 *g* (10 min) to yield a lysosome-enriched granular pellet and a combined microsomal-cytosol fraction; the latter was further fractionated by centrifugation at 100000 *g* for 60 min to yield the individual microsomal and cytosolic (soluble) fractions. Each fraction was analysed for cystine content and for acid hydrolase (α -mannosidase) activity.

Subcellular fractionation of cystine-loaded normal fibroblasts

Normal fibroblasts (GM 3349) were loaded in monolayer with free cystine by incubation with 0.5 mM or 1.0 mM non-radioactive L-cystine dimethyl ester for 60 min at 37 °C in complete medium. Cells were harvested and ruptured by low-pressure nitrogen cavitation in 0.25 M-sucrose/10 mM-Hepes (pH 7.0) containing or not containing 2 mM-NEM, and subjected to the centrifugation protocol described above. As before, fractions were assayed for cystine and for acid hydrolase (hexosaminidase) activity.

Gradient fractionation of cystinotic and I-cell-disease fibroblast homogenates in colloidal silica

Cystinotic and I-cell-disease fibroblasts were labelled by exposure to $[^{35}\text{S}]$ cystine for 48 h as described above. After rupture by nitrogen cavitation, the post-nuclear supernatants were subjected to gradient fractionation in colloidal silica as described by Rome *et al.* (1979). After removal of a small portion of each eluate fraction for determination of lysosomal enzyme activity, the remainder was treated with 5% trichloroacetic acid, and the precipitated protein and silica were removed by centrifugation (1000 *g*, 5 min). A suitable portion of each supernatant fraction was then subjected to high-voltage electrophoresis as described above.

RESULTS AND DISCUSSION

Subcellular localization of cystine

Because cystine clearance from whole cells and their granular fractions was considered to reflect clearance specifically from lysosomes, it was essential to demonstrate that the cystine content of fibroblasts was concentrated within the lysosomes. In cystinotic fibroblasts, we measured the clearance of endogenous cystine, which is well known to be contained within the lysosomal compartment (Schulman *et al.*, 1969). For I-cell-disease fibroblasts we also relied on endogenous cystine accumulation to measure lysosomal cystine clearance, since preliminary experiments, in agreement with those of Steinhilber *et al.* (1984a), showed that cystine dimethyl ester was only poorly hydrolysed to free cystine in I-cell-disease fibroblasts. Therefore, to demonstrate that most of the cystine occurring naturally within I-cell-disease fibroblasts was intralysosomal, we fractionated these cells by differential centrifugation into their subcellular components. As with similarly fractionated cystinotic cells, the free cystine co-sedimented predominantly with

Table 2. Distribution of free cystine in subcellular fractions of normal fibroblasts exposed to cystine dimethyl ester

Normal fibroblasts (GM 3349) were incubated with 1 mM- (Expt. 1) or 0.5 mM- (Expt. 2) L-cystine dimethyl ester for 60 min at 37 °C, washed with phosphate-buffered saline and disrupted by nitrogen cavitation in 0.25 M-sucrose. Subcellular components were isolated by differential centrifugation and assayed for cystine content and for hexosaminidase activity as described in the Experimental section. Fractions: Nuc, nuclear; Lys, lysosomal; Mic, microsomal; Supt, 100 000 g supernatant fraction. In Expt. 2, cells were ruptured in the presence of 2 mM-NEM.

Expt. no.	Fraction	Total half-cystine		Total hexosaminidase	
		(nmol)	(%)	(units)	(%)
1	Nuc	3	8	8	10
	Lys	21	55	71	85
	Mic	3	8	3	4
	Supt	11	29	2	2
2	Nuc	0.5	6	9	6
	Lys	5.9	66	124	83
	Mic-Supt	2.6	29	16	11

Table 3. Clearance of free cystine in normal, cystinotic and I-cell-disease fibroblasts

Cells were labelled as described in the text. Values of $t_{\frac{1}{2}}$ were calculated from the slope of the linear regression of $\ln(\text{nmol of half-cystine/mg of protein})$ versus time (min). Symbols in parentheses denote cell strains.

Expt. no.	Time (min)	Half-cystine (nmol/mg of protein)		
		Normal (Sc)	Cystinotic (Ha)	I-cell (Pe)
1	0	12.1	33.4	5.7
	30	5.6	27.9	6.5
	60	3.0	30.5	5.7
	120	1.7	28.7	5.4
	$t_{\frac{1}{2}}$ (min) . . .	43	> 500	> 500
		(GM 495)	(El)	(GM 3066)
2	0	3.10	12.7	7.4
	30	0.60	11.7	7.4
	60	0.35	12.3	9.1
	120	0.18	11.0	7.4
	$t_{\frac{1}{2}}$ (min) . . .	32	> 500	> 500

lysosomal enzyme activity (Table 1). These findings were further confirmed by the results of colloidal-silica-gradient centrifugation (Fig. 1), which again showed excellent correlation between free cystine and lysosomal enzyme activity in eluate fractions from both cystinotic and I-cell-disease fibroblasts.

To load normal fibroblasts with cystine, whole cells were exposed to 0.5 mM-cystine dimethyl ester (see the Experimental section). High-voltage paperelectrophoresis experiments using ^{35}S -labelled cystine dimethyl ester

demonstrated, as previously reported (Steinherz *et al.*, 1982a), virtually complete hydrolysis to [^{35}S]cystine, with some free cystine detected as the [^{35}S]cystine-NEM adduct (results not shown). Subcellular fractionation by differential centrifugation in 0.25 M-sucrose showed that most of the cystine within normal fibroblasts exposed to cystine dimethyl ester was closely associated with lysosomal enzyme activity (Table 2).

Therefore we considered that clearance of cystine from whole normal, cystinotic and I-cell-disease fibroblasts reflected lysosomal cystine clearance indirectly, whereas clearance from granular fractions reflected lysosomal cystine clearance directly.

Cystine clearance from whole fibroblasts

The loss of cystine from normal, cystinotic and I-cell-disease fibroblasts was demonstrated by calculating a $t_{\frac{1}{2}}$ for the clearance of cystine/mg of protein over a 2 h period (Table 3). The disposal of free cystine by normal cells occurred at a rapid rate ($t_{\frac{1}{2}} = 30\text{--}40$ min) compared with prolonged rates for both cystinotic and I-cell-disease fibroblasts ($t_{\frac{1}{2}} > 500$ min). Disposal rates for normal and cystinotic fibroblasts are similar to those reported for intact leucocytes and fibroblasts (Steinherz *et al.*, 1982a, 1984a). Comparable amounts of intracellular cystine were seen in the normal and I-cell-disease fibroblasts at zero time of clearance (Table 3); indeed, in Expt. 1, the intracellular amount of cystine in the normal cells was actually higher than that for I-cells. The importance of utilizing comparably loaded cells for comparison of $t_{\frac{1}{2}}$ values for cystine disposal has been emphasized previously (Steinherz *et al.*, 1982a).

Cystine clearance from isolated granular fractions

This was investigated in a manner identical with that for whole fibroblasts. When cystine content was expressed as nmol of half-cystine per unit of hexosaminidase, acid phosphatase or protein, normal granular fractions lost cystine with a $t_{\frac{1}{2}}$ of approx. 41 min (Table 4). For cystinotic granular fractions, there was no loss of cystine in 50 min, whereas I-cell granular fractions exhibited $t_{\frac{1}{2}}$ of over 100 min. Loading of the I-cell granular fractions was substantially less than that for the normal granules; this eliminated the possibility that the $t_{\frac{1}{2}}$ for the I-cells was prolonged because saturation of a carrier had caused a maximal velocity of cystine egress to be reached. Note that cystine loading expressed as nmol of half-cystine/unit of hexosaminidase was very high for the I-cell granules; this was due to lysosomal hexosaminidase deficiency in these cells.

To determine whether different I-cell strains exhibited the same defect in granular-fraction cystine egress, the experiment was performed four times with three different normal, cystinotic and I-cell-disease fibroblast strains. Mean loadings ($\pm$ s.d.) at zero time of egress were 0.39 ± 0.52 , 0.52 ± 0.28 and 1.01 ± 0.75 nmol of half-cystine/unit of hexosaminidase for normal, cystinotic and I-cell granular fractions respectively. With cystine expressed per mg of granular-fraction protein, the composite $t_{\frac{1}{2}}$ values for all normal, cystinotic and I-cell strains were 43 min, ∞ and 108 min respectively (Fig. 2). Virtually identical results were obtained when cystine was expressed per unit of hexosaminidase or acid phosphatase. The $t_{\frac{1}{2}}$ values for normal and cystinotic fibroblast granular fractions closely resembled those for granular fractions isolated from polymorphonuclear leucocytes,

Table 4. Cystine loss from normal, cystinotic and I-cell-disease fibroblast granular fractions

Data were from a single representative experiment with cystine, hexosaminidase (Hex), acid phosphatase (AP) and protein determinations performed in duplicate for each time point. Only the normal cells were exposed to cystine dimethyl ester (0.5 mM), as in the Experimental section. Cystine loading per unit of hexosaminidase is elevated in the I-cell granular fractions because of the hexosaminidase deficiency in I-cell lysosomes.

Cells	Time (min)	Cell rupture (%)	Half-cystine (nmol) per:		
			Unit of Hex	Unit of AP	mg of protein
Normal (GM 3349)	0	2.9	0.36	238	15.3
	10	8.0	0.36	217	11.7
	20	9.5	0.23	164	8.0
	30	12.4	0.24	149	9.9
	40	25.6	0.17	121	7.4
	50	21.5	0.15	107	6.7
	$t_{\frac{1}{2}}$ (min) . . .		37	41	46
	Correlation coefficient . . .		0.96	0.99	0.91
Cystinotic (Ha)	0	23.4	0.29	384	27.0
	10	25.0	0.29	387	30.1
	20	32.0	0.30	410	26.7
	30	32.2	0.31	444	30.3
	40	44.2	0.27	373	30.0
	50	46.6	0.29	440	34.4
	$t_{\frac{1}{2}}$ (min) . . .		1336	∞	∞
	Correlation coefficient . . .		0.21	—	—
I-cell (GM 1586)	0	21.2	1.28	40	5.8
	10	23.6	1.44	33	5.7
	20	28.7	1.00	31	4.2
	30	17.9	1.20	30	6.0
	40	33.7	1.18	31	4.4
	50	35.1	1.05	26	4.7
	$t_{\frac{1}{2}}$ (min) . . .		173	102	165
	Correlation coefficient . . .		0.57	0.90	0.50

which were 46 min (normal) and ∞ (cystinotic) (Gahl *et al.*, 1982b).

To verify that exposure to cystine dimethyl ester itself did not cause cystine leakage, a cystinotic strain was exposed to 0.20 mM-cystine dimethyl ester. The $t_{\frac{1}{2}}$ for cystine egress from the isolated granular fraction was infinite.

When rates of cystine egress from granular fractions were expressed as velocities (Gahl *et al.*, 1982a), the mean values ($\pm$ s.d.) for normal, cystinotic and I-cell granular fractions were 5.3 ± 1.6 , 1.1 ± 0.7 and 5.1 ± 4.1 pmol of half-cystine/unit of hexosaminidase per min respectively. The last value represents a very low rate of cystine loss, since hexosaminidase activity is very low in I-cell-disease fibroblasts. In fact, the ratio of acid phosphatase to hexosaminidase activity in I-cell granular fractions was 15 times that in cystinotic and normal fibroblast granular fractions.

These results verify that cultured human fibroblasts exhibit the lysosomal cystine-transport system first demonstrated in human polymorphonuclear-leucocyte granular fractions (Gahl *et al.*, 1982a,b, 1983) and also in lymphocytes (Jonas *et al.*, 1982). The prolonged clearance of cystine from cystinotic fibroblast granular fractions also agrees with reports that the defect in cystinosis can be shown in cultured fibroblasts (Pisoni *et al.*, 1985; Smith *et al.*, 1985).

Although protein turnover could conceivably contribute to measured intracellular or intralysosomal cystine contents during clearance or egress, it is unlikely to have been a factor in our experiments, for several reasons. Firstly, only very small amounts of cyst(e)ine can be expected to become incorporated into protein during the minimal incubation time of normal cells with cystine dimethyl ester (30–60 min), and only very small amounts of cyst(e)ine should be produced from catabolism during the clearance or egress period. In this connection, it is pertinent to note that normal fibroblasts exhibit a steady-state content of approx 0.5 nmol of half-cystine/mg of granular-fraction protein (F. Tietze, J. DeB. Butler & L. H. Rome, unpublished work); this represents only 1–10% of that generally found in the granular fractions from our mutant or loaded normal cells (cf. Table 4). This amount of protein-derived cystine, which should not be influenced by cell exposure to cystine dimethyl ester, would not be expected to contribute significantly to the intragranular content of free cystine during efflux. Secondly, the total amount of granular fraction free cystine (i.e. inside + outside) did not increase substantially during the egress period (results not shown), indicating that release of free cystine from bound forms of the amino acid did not occur. The normal and mutant cells behaved identically in this respect, consistent with our earlier observations with leucocyte granular fractions (Gahl *et*

Fig. 2. Cystine loss from granular fractions isolated from normal, cystinotic and I-cell fibroblasts

Samples were removed from incubations of cystine-loaded granular fractions and centrifuged. Cystine content/mg of protein remaining in the pellet at each time point was expressed as % of the initial value. Straight lines were drawn by the least-squares method; half-times were derived from the slopes. Points represent means and bars S.E.M. for four experiments involving three normal (○), three cystinotic (●) and three I-cell (▲) fibroblast strains.

al., 1982a). Thirdly, any spurious increase in free intralysosomal cystine during normal efflux (e.g. from proteolysis) should prolong the half-time of cystine efflux relative to that observed in the corresponding I-cell fraction, in which proteolysis is impaired. Thus differences in half-time values of cystine efflux from the granular fractions of these two cell types would be minimized rather than maximized.

Our data show a clear impairment of cystine egress from I-cell fibroblast granular fractions (Table 4, Fig. 2). This is in marked contrast with the report by Steinherz *et al.* (1984b), who claim to have observed normal rapid loss of cystine from I-cell-disease fibroblasts exposed to the dimethyl ester. In attempting to reconcile these disparate conclusions, several considerations should be borne in mind. Firstly, our experiments with intact I-cell-disease fibroblasts involved quantitative determination of the loss of endogenous intralysosomal cystine, whereas Steinherz *et al.* (1984b) qualitatively assessed loss of cystine from an undefined subcellular locus in esterase-deficient cells with initially high contents of unhydrolysed dimethyl ester. Secondly, it is apparent from the data of Steinherz *et al.* (1984b) that neither graphically nor numerically were these authors able to distinguish clearly between the rates of disappearance of cystine in normal and cystinotic cells (cf. especially their Fig. 1); under these circumstances their conclusion that cystine disappearance from I-cell-disease fibroblasts resembles one (normal) more than the other (cystinotic) may not be warranted.

The fact that, among the free amino acids, cystine

alone accumulates in I-cell lysosomes (Tietze & Butler, 1979) is intriguing in itself. The additional finding that the cystine accumulates because of impaired lysosomal cystine transport, as in nephropathic cystinosis, invites speculation as to the mechanism of the defect in mucopolipidosis II. In the light of findings of abnormal synthesis of lysosomal glycoproteins in I-cell fibroblasts (Hasilik & Neufeld, 1980b; Hasilik *et al.*, 1981; Reitman *et al.*, 1981; Waheed *et al.*, 1982), it seems possible that I-cells may display a defectively packaged cystine carrier glycoprotein which lacks essential 6-phosphomannosyl residues. Alternatively, impaired carrier function could be the result of inadequate proteolytic processing (Hasilik & Neufeld, 1980a,b) of an inactive precursor form of the carrier owing to abnormally low activity of appropriate lysosomal proteinases (Williams *et al.*, 1982). Although the specific cause of abnormal cystine-carrier function in I-cell-disease fibroblasts remains unknown, study of this defect should enhance our knowledge not only of the cystine carrier itself but also of how integral lysosomal membrane proteins in general arrive at their destination and function.

In a paper appearing after acceptance of our manuscript, Greene *et al.* (1985) have also reported decreased rates of efflux of free cystine from lysosome-rich granular fractions of cystinotic and I-cell-disease fibroblasts.

REFERENCES

- Gahl, W. A. & Tietze, F. (1985) *Biochem. J.* **228**, 263–267
- Gahl, W. A., Bashan, N., Tietze, F., Bernardini, I. & Schulman, J. D. (1982a) *Science* **217**, 1263–1265
- Gahl, W. A., Tietze, F., Bashan, N., Steinherz, R. & Schulman, J. D. (1982b) *J. Biol. Chem.* **257**, 9570–9575
- Gahl, W. A., Tietze, F., Bashan, N., Bernardini, I., Raiford, D. & Schulman, J. D. (1983) *Biochem. J.* **216**, 393–400
- Gahl, W. A., Bashan, N., Tietze, F. & Schulman, J. D. (1984a) *Am. J. Hum. Genet.* **36**, 277–282
- Gahl, W. A., Tietze, F., Butler, J. & Bernardini, I. (1984b) *Pediatr. Res.* **18**, 221A
- Goldman, R. & Kaplan, A. (1973) *Biochim. Biophys. Acta* **318**, 205–216
- Greene, A. A., Jonas, A. J., Harms, E., Smith, M. L., Pellett, O. L., Bump, E. A., Miller, A. L. & Schneider, J. A. (1985) *Pediatr. Res.* **19**, 1170–1174
- Hall, C. W., Liebaers, I., DiNatale, P. & Neufeld, E. F. (1978) *Methods Enzymol.* **50**, 439–456
- Hanai, J., Leroy, J. & O'Brien, J. S. (1971) *Am. J. Dis. Child.* **122**, 34–38
- Hasilik, A. & Neufeld, E. F. (1980a) *J. Biol. Chem.* **255**, 4937–4945
- Hasilik, A. & Neufeld, E. F. (1980b) *J. Biol. Chem.* **255**, 4946–4950
- Hasilik, A., Waheed, A. & von Figura, K. (1981) *Biochem. Biophys. Res. Commun.* **98**, 761–767
- Hollemans, M., Elferink, R. O., DeGroot, P. G., Strijland, A. & Tager, J. M. (1981) *Biochim. Biophys. Acta* **643**, 140–151
- Jonas, A. J., Bump, E. A., Harms, E., Pellett, O. L. & Schneider, J. A. (1981) *Pediatr. Res.* **15**, 563
- Jonas, A. J., Smith, M. L. & Schneider, J. A. (1982) *J. Biol. Chem.* **257**, 13185–13188
- Kaplan, A., Achord, D. T. & Sly, W. S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2026–2030
- Leroy, J. G., Ho, M. W., MacBrinn, M. C., Zielke, K., Jacob, J. & O'Brien, J. S. (1972) *Pediatr. Res.* **6**, 752–757
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

- Neufeld, E. F. & McKusick, V. A. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M. S., eds.), 5th edn., pp. 778–787, McGraw-Hill, New York
- Oshima, R. G., Willis, R. C., Furlong, C. E. & Schneider, J. A. (1974) *J. Biol. Chem.* **249**, 6033–6039
- Pisoni, R. L., Thoene, J. G. & Christensen, H. N. (1985) *J. Biol. Chem.* **260**, 4791–4798
- Reeves, J. P. (1979) *J. Biol. Chem.* **254**, 8914–8921
- Reitman, M. L. & Kornfeld, S. (1981a) *J. Biol. Chem.* **256**, 4275–4281
- Reitman, M. L. & Kornfeld, S. (1981b) *J. Biol. Chem.* **256**, 11977–11980
- Reitman, M. L., Varki, A. & Kornfeld, S. (1981) *J. Clin. Invest.* **67**, 1574–1579
- Rome, L. H., Garvin, A. J., Allietta, M. M. & Neufeld, E. F. (1979) *Cell* **17**, 143–153
- Schneider, J. A. & Schulman, J. D. (1983) in *Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M. S., eds.), 5th edn., pp. 1844–1866, McGraw-Hill, New York
- Schulman, J. D., Bradley, K. H. & Seegmiller, J. E. (1969) *Science* **166**, 1152–1153
- Sly, W. S. & Fischer, H. G. (1982) *J. Cell. Biochem.* **18**, 67–85
- Smith, M. L., Greene, A. A. & Schneider, J. A. (1985) *Pediatr. Res.* **19**, 254A
- Steinherz, R., Tietze, F., Gahl, W. A., Triche, T. J., Chiang, H., Modesti, A. & Schulman, J. D. (1982a) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4446–4450
- Steinherz, R., Tietze, F., Raiford, D., Gahl, W. A. & Schulman, J. D. (1982b) *J. Biol. Chem.* **257**, 6041–6049
- Steinherz, R., Makov, N., Narinsky, R., Meidan, B. & Kohn, G. (1984a) *Clin. Chim. Acta* **141**, 119–126
- Steinherz, R., Makov, N., Narinsky, R., Meidan, B. & Kohn, G. (1984b) *Enzyme* **32**, 126–130
- Tietze, F. (1976) *Pediatr. Res.* **10**, 372
- Tietze, F. & Butler, J. D. (1979) *Pediatr. Res.* **13**, 1350–1355
- Waheed, A., Pohlman, R., Hasilik, A., von Figura, K., van Elsen, A. & Leroy, J. G. (1982) *Biochem. Biophys. Res. Commun.* **105**, 1052–1058
- Wenger, D. A., Sattler, M., Clark, C. & Wharton, C. (1976) *Life Sci.* **19**, 413–420
- Williams, J. C., Weinstein, D. B., Miller, A. L. & Steinberg, D. (1982) *Biochem. J.* **202**, 183–190

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