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Cystine accumulation and clearance by normal and cystinotic leukocytes exposed to cystine dimethyl ester

(cystinosis/lysosomes/transport/biological membrane/disulfides and thiols)

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ABSTRACT **Upon exposure to 0.25 mM cystine dimethyl ester, normal and cystinotic leukocytes accumulate substantially more intracellular cystine than is present endogenously in cystinotic cells. Leukocytes loaded by exposure to cystine dimethyl ester may have abnormally lucent and distended lysosomes, and the cystine is compartmentalized within the granular fraction of the cells. After the cells are exposed to cystine dimethyl ester, cystine clearance from normal leukocytes is much faster than from cystinotic cells. The ratios of labeled cysteine-*N*-ethylmaleimide to cystine are also greater in normals than in cystinotics 60 min after termination of loading. No overlap in ranges of cystine clearance half-times or cysteine-*N*-ethylmaleimide to cystine ratios was observed in normal compared to cystinotic leukocytes. Limited experiments with fibroblasts exposed to cystine dimethyl ester suggest a correspondingly prolonged cystine clearance for cystinotic cells. These experiments provide evidence for defective clearance of cystine from cystinotic lysosomes *in situ*.**

In classical cystinosis, an autosomal recessive disorder (1, 2), progressive kidney disease usually leads to death before age 10 unless renal transplantation is performed. Many cystinotic tissues, including leukocytes and cultured fibroblasts, have greatly increased concentrations of free cystine (1–4), with cystine compartmentalization in lysosomes (1, 2, 5–7).

Although the fundamental metabolic defect remains unknown, one possibility is that cystinotic cells lack a mechanism for disposal of cystine from their lysosomes. However, there is no direct evidence for defective efflux of cystine from cystinotic lysosomes, despite attempts to assess transcellular uptake or efflux in whole cystinotic cells in comparison with normal cells (8, 9). Examinations of subcellular transport have been limited by the inability to load normal cells or lysosomes to cystine concentrations approximating the cystinotic.

Goldman and Kaplan (10) and Reeves (11) have demonstrated that certain amino acid methyl esters can be used to load isolated rat liver lysosomes with high concentrations of the corresponding amino acid. Apparently, the methyl esters penetrate lysosomal membranes and are hydrolyzed by esterases to free amino acids, which accumulate because of slow efflux from the lysosomes. Using radioactive amino acid methyl esters, Reeves characterized the efflux of certain amino acids from rat liver lysosomes (11). We have used related techniques to study amino acid efflux from isolated normal and cystinotic human leukocyte lysosomes (12–14).

In this report, we demonstrate that intact normal and cystinotic human leukocytes incubated with cystine dimethyl ester accumulate cystine, primarily within lysosomes, in concentrations far exceeding those present endogenously in cystinotic cells. Loaded cystinotic leukocytes exhibited retarded cystine clearance when compared to normal cells. These observations strongly suggest that cystinotic lysosomes *in situ* are defective in their capacity to eliminate cystine.

MATERIALS AND METHODS

Cystine dimethyl ester was purchased from Sigma. Radioactive cystine dimethyl ester was prepared by methylation of radioactive cystine ([^{35}S]cystine, ≈150 mCi/mmol, Amersham; 1 Ci = 3.7×10^{10} becquerels) in 3 M anhydrous methanolic HCl (11–13).

Leukocytes (heparinized blood) were prepared as described (3, 12). The washed leukocytes were suspended in Hanks' balanced salt solution (Ca- and Mg-free), pH 7.0, and mixed into tubes containing cystine dimethyl ester (final concentration 0.25 mM). Ester was introduced into the tubes as a methanolic solution and dried with N_2 before cells were added. Usually each tube contained leukocytes representing 3–5 ml of blood suspended in 2 ml of Hanks' solution.

The cells were incubated with cystine dimethyl ester at 37°C for various time periods with gentle agitation every 10 min, then centrifuged at ≈600 × *g* for 3 min. The cell pellets were resuspended in 4 ml of cold phosphate-buffered saline at pH 7.0 and centrifuged, and the final pellets were resuspended in 2 ml of Hanks' solution at 37°C. The clearance period then involved continued 37°C incubation in Hanks' solution. At appropriate times during the loading or clearance periods cell suspensions were processed for various studies.

For direct measurement of intracellular cystine content, leukocytes were washed and centrifuged three times in 4 ml of phosphate-buffered saline at 4°C and acidified with sulfosalicylic acid (final concentration 2–4%), and cystine was determined on a Beckman 121M amino acid analyzer. Quantitation of the intracellular content of several amino acids compared to controls incubated in the absence of the dimethyl ester or compared to pretreatment (nonloaded) samples provided one assessment of leukocyte integrity. In addition, viability was also determined by exclusion of 0.4% trypan blue.

For electron microscopy, cells were fixed in Palay's fixative (1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.05% $CaCl_2$, pH 7.4). Each specimen was divided into two parts, one processed by routine techniques (15) and the other incubated with 0.03% diaminobenzidine/0.018% H_2O_2 for detecting peroxidase activity before treatment with OsO_4 (16). The cells were washed and centrifuged before

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Abbreviations: MalNEt, *N*-ethylmaleimide; PMN, polymorphonuclear neutrophils.

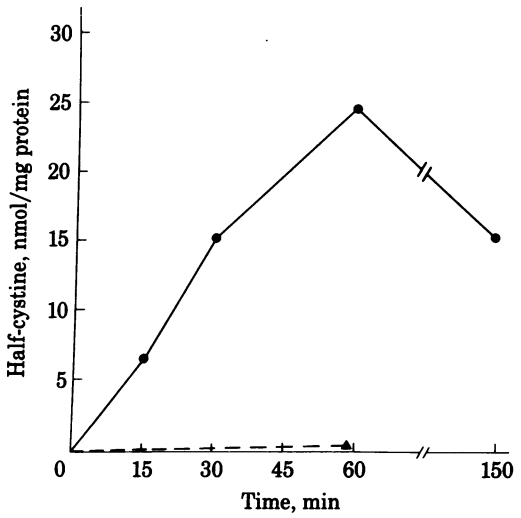

FIG. 1. Normal leukocytes were exposed to 0.25 mM cystine dimethyl ester (●) or 0.5 mM (▲) cystine and then processed as described in the text. Cystine content was determined by amino acid analysis.

and after the diaminobenzidine and OsO_4 procedures, the pellets were embedded in Maraglas, and thin sections were examined in a Philips 201 electron microscope. Sections were photographed after uranyl acetate and lead citrate heavy metal staining except that cells in which myeloperoxidase activity was studied were photographed unstained, after OsO_4 treatment.

Lysosomal circumferences were measured with a Hewlett–Packard digitizer and computer (17). Percentages of abnormal lysosomes in individual cells were determined by comparing the number of lysosomes with lucent areas, with or without distention, to their total number.

For serial assessment of cellular content of cystine (half-time for clearance, $t_{1/2}$) and certain intracellular metabolites, leukocytes from 3–5 ml of blood were loaded in 2 ml of Hanks' solution containing 0.25 mM [^{35}S]cystine dimethyl ester (final specific activity ≈50 mCi/mmol) for 30 min at 37°C, then washed and incubated in fresh Hanks' solution as described above. At least three incubations were set up on samples from each patient or control. During clearance, tubes were harvested every 15–30 min for at least 1 hr. Cells were centrifuged, washed three times in 4 ml of cold phosphate-buffered saline, placed in sodium phosphate, pH 7.0, with 10 mM *N*-ethylmaleimide (MalNEt), disrupted by freezing and thawing (acetone and dry ice), and, after 15 min at 25°C, acidified with sulfosalicylic acid (final 2%). Distribution of radioactivity in the supernatant was determined by high-voltage electrophoresis in 7.4% formic acid on a Gilson model D electrophoresis apparatus, using Whatman 3MM paper, 4,000 volts at 17°C, and 20- to 60-min run time. Radioactivity in areas corresponding to cystine, cysteine-MalNEt, glutathione-MalNEt, and cystine monomethyl and dimethyl esters was determined by using a scintillation counter. Cystine clearance $t_{1/2}$ was calculated from a linear regression line fitted to a semilogarithmic plot of cystine radioactivity against time with at least three data points between 0 and 60 min; counts were corrected for protein content in each sulfosalicylic acid precipitate (18).

RESULTS

Normal leukocytes incubated at 37°C with 0.25 mM cystine dimethyl ester accumulated cystine for approximately 60–120 min (Fig. 1). The final cystine concentration of loaded normal leukocytes (usually 25–30 nmol of half-cystine per mg of cell protein) was 5- to 50-fold greater than the endogenous cystine levels of the cystinotic cells studied. For example, cystine levels of leukocytes loaded with 0.25 mM cystine dimethyl ester for 60 min at 37°C, determined by amino acid analyzer, were 94.8 nmol of half-cystine per mg of protein for a cystinotic preparation (pretreatment 2.32) and 40.2 for a normal sample (pretreatment 0.03).

The electron microscopy of the polymorphonuclear neutrophils (PMNs) is illustrated in Fig. 2. In a representative experiment with normal PMNs unexposed to cystine dimethyl ester only a small percentage (<3%; Table 1) of neutrophil-specific granules were enlarged and devoid of uniformly electron-dense content. After exposure to dimethyl ester (37°C, 30 min) 14% of the granules became abnormal, but this was rapidly reversed when exogenous ester was removed. Unexposed PMNs from a cystinosis patient contained more abnormal lysosomes (≈10%), which increased 3-fold on exposure to dimethyl ester (Fig. 2*B*; Table 1). In marked contrast to normal PMNs, the percentage of abnormal granules remained elevated after 1 hr of clearance at 37°C. The abnormal granules were clearly lysosomal (myeloperoxidase positive) (Fig. 2*C*). The peroxidase-negative granules, presumably neutrophil-specific secondary (alkaline phosphatase) granules, always appeared normal.

Although slightly larger initially, mean granule circumference was substantially larger in the cystinotic PMNs when they were exposed to cystine dimethyl ester, and the granules remained comparably enlarged after 1-hr clearance (Table 1); lysosomal size in normal PMNs remained constant throughout.

The electron microscopic studies suggested that the bulk of cystine had accumulated within lysosomes. This was confirmed by measurement of the cystine content in subcellular fractions of normal leukocytes loaded with 0.25 mM cystine dimethyl ester at 37°C. In a representative experiment, after 15 min of loading cells were washed and sonicated for 10 sec (Branson sonicator with microtip) (3). Postnuclear 165,000 × *g*·min granular and supernatant fractions were prepared, and allowed to react with 10 mM MalNEt, and cystine was determined. Cystine concentration in the granular fraction (21.3 nmol of half-cystine per mg of protein) was substantially higher than in the supernatant fraction (3.19 nmol of half-cystine per mg). This compartmentalization of intracellular cystine in normal leukocytes exposed to cystine dimethyl ester corresponds to the lysosomal compartmentalization of cystine in untreated cystinotic leukocytes (1–3, 5).

Incubation of leukocytes with cystine dimethyl ester at concentrations as high as 1 mM for 60 min at 37°C failed to alter

Table 1. Percentage of abnormal neutrophil granules and circumference of lysosomes measured by electron microscopy

PMNs	Abnormal neutrophil granules, %			Circumference of lysosomes, nm		
	Unexposed	After 30-min exposure	After 1-hr clearance	Unexposed	After 30-min exposure	After 1-hr clearance
Cystinotic	9.2	26.4	26.8	1,100 ± 281 (34)	1,389 ± 439 (39)	1,504 ± 478 (47)
Normal	2.8	13.6	2.4	886 ± 252 (31)	847 ± 218 (33)	845 ± 206 (30)

A single cystinotic and a single normal cell source were used. For measurement of circumferences, only myeloperoxidase-positive vacuoles were counted. Parentheses indicate number of vacuoles measured and ± indicates SD. Differences between cystinotic and normal in each column are signficant ($P < 0.05$).

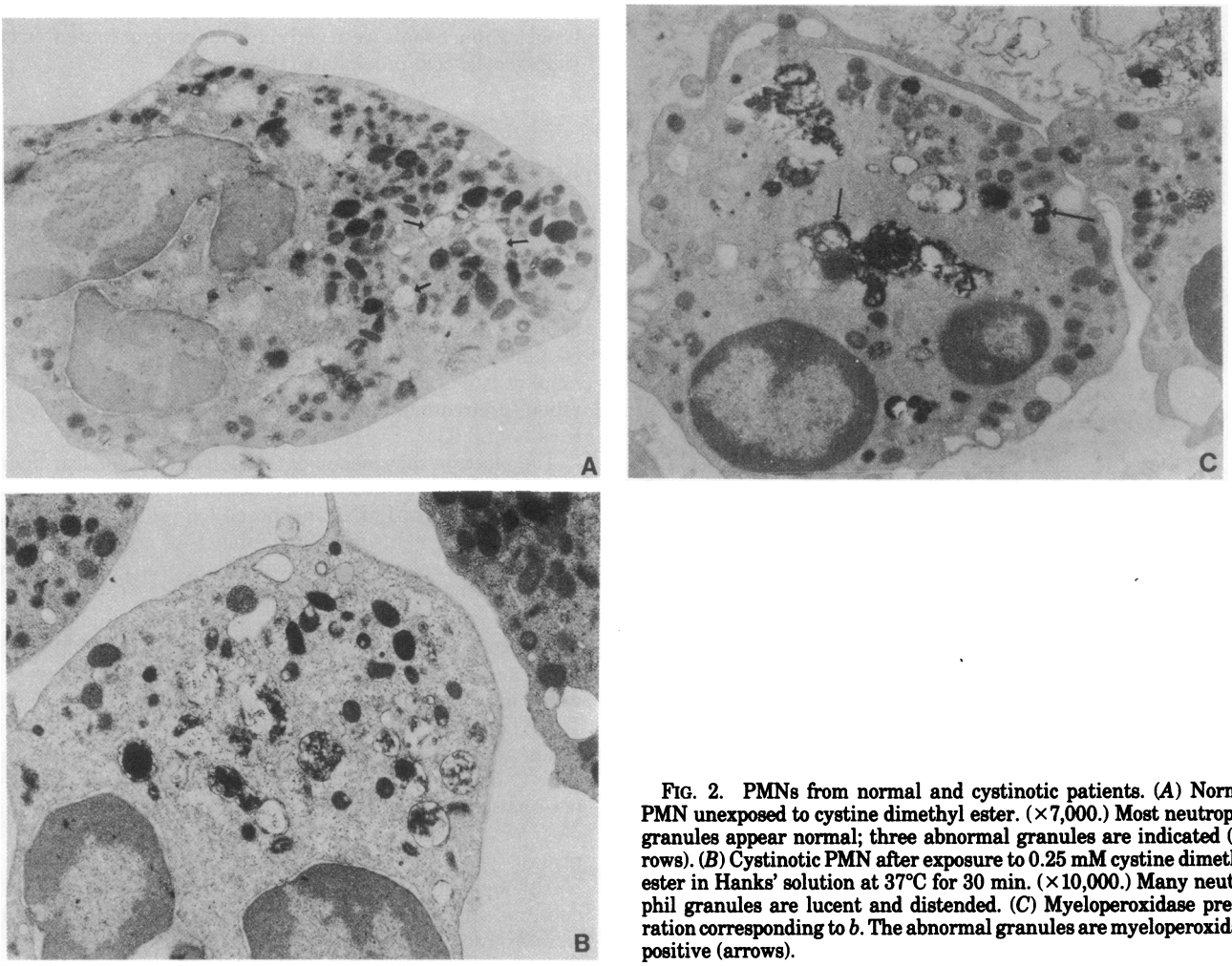

FIG. 2. PMNs from normal and cystinotic patients. (*A*) Normal PMN unexposed to cystine dimethyl ester. (×7,000.) Most neutrophil granules appear normal; three abnormal granules are indicated (arrows). (*B*) Cystinotic PMN after exposure to 0.25 mM cystine dimethyl ester in Hanks' solution at 37°C for 30 min. (×10,000.) Many neutrophil granules are lucent and distended. (*C*) Myeloperoxidase preparation corresponding to *b*. The abnormal granules are myeloperoxidase positive (arrows).

the concentration of amino acids other than cystine within the washed leukocytes and did not decrease the percentage of trypan blue-excluding cells (data not shown). Incubation of normal leukocytes with 0.5 mM cystine did not significantly increase intracellular cystine (Fig. 1).

When cystinotic and normal leukocytes were exposed to 0.25 mM radioactive cystine dimethyl ester for 30 min at 37°C, then washed and incubated for 1 hr, cystinotic cells lost radioactive cystine more slowly than normal during clearance (Fig. 3). In cystinotic cells, radioactivity in cysteine-MalNEt and glutathione-MalNEt initially was less than in the cystine pool and decreased rapidly. [Detailed electrophoretic analyses demonstrated that this combined peak (a and b) in loaded cells was primarily cysteine-MalNEt.] In normal cells, radioactivity in cysteine-MalNEt initially approximated that in the cystine pool and also decreased rapidly.

In 19 normal and 10 cystinotic leukocyte preparations, cystinotic cells had much longer mean $t_{1/2}$ values ($P < 0.01$) and lower cysteine-MalNEt to cystine ratios ($P < 0.001$) with no overlap in values between the two groups (Table 2).

These results cannot be accounted for by greater dilution of radioactive cystine with unlabeled cystine in the cystinotic samples because *both* cystinotic and normal leukocytes were hyperloaded with radioactive ester to cystine levels severalfold greater than the endogenous (unlabeled) cystine content of the cystinotic cells. Cystinotic cells had greatly prolonged cystine clearance whether or not the patients were receiving cysteamine (19); cystinotic samples 4 and 10 in Table 2 are from the

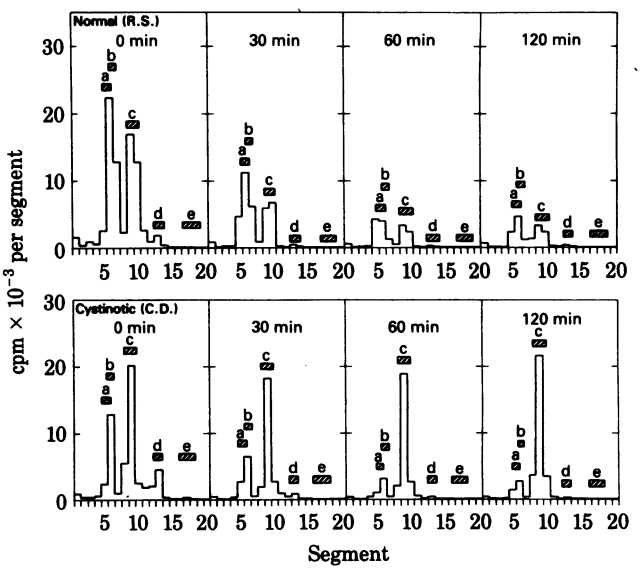

FIG. 3. Clearance of [^{35}S]cystine from normal (R.S.) and cystinotic (C.D.) leukocytes. Leukocytes were exposed to 0.25 mM [^{35}S]cystine dimethyl ester for 30 min at 37°C, washed, and resuspended in Hanks' solution at 37°C. Samples of the resultant suspension were withdrawn at the indicated times, washed, and treated successively with 10 mM MalNEt and sulfosalicylic acid; aliquots of the protein-free supernatants were subjected to electrophoretic analysis. Hatched segments: a, glutathione-MalNEt; b, cysteine-MalNEt; c, cystine; d, cystine monomethyl ester; e, cystine dimethyl ester.

Table 2. Cystine $t_{1/2}$ and 60-min cysteine-MalNEt to cystine ratios of normal and cystinotic leukocytes loaded with cystine dimethyl ester and their pretreatment cystine content

Sample no.	Name	$t_{1/2}$, min	Cysteine-MalNEt/ cystine	Half-cystine,* nmol/mg cell protein
		Normal		
1	F.T.	33.8	2.13	—
2	R.S.	28.5	1.66	0.05
3	T.A.	34.6	1.63	—
4	H.A.	31.5	1.57	—
5	J.A.	48.1	0.92	—
6	D.R.	47.1	1.79	—
7	P.B.	47.4	1.89	—
8	J.S.	36.8	1.48	0.07
9	B.B.	47.1	1.47	<0.27
10	N.V.	57.7	2.12	0.086
11	Y.E.	32.8	1.50	<0.26
12	J.E.	58.7	1.18	<0.15
13	L.Y.	34.6	1.43	0.034
14	K.A.	76.2	1.86	0.084
15	C.A.	28.7	1.90	<0.28
16	V.S.	21.9	3.35	0.20
17	N.B.	40.5	1.67	0.026
18	J.O.	60.3	1.35	—
19	T.O.	45.5	1.41	0.041
	Mean	42.7 ± 3.1	1.70 ± 0.11	
		Cystinotic		
1	C.D.	>500	0.19	—
2	B.M.	103	0.16	—
3	S.G.†	>500	0.21	3.6
4	D.B.	165	0.25	0.51
5	S.W.	123	0.26	0.23
6	B.R.	451	0.29	0.98
7	P.H.	80	0.22	0.74
8	P.M.	>500	0.26	—
9	K.D.	>500	0.19	—
10	D.B.†	315	0.26	3.2
	Range 80–>500		Mean 0.23 ± 0.15	

Means are presented ±SEM.
* Endogenous level before loading. All cystinotic patients except † were receiving oral cysteamine therapy, explaining the relatively low initial cystine levels.

same patient receiving cysteamine and no longer receiving it, respectively, and sample 3 was from a different cystinosis patient no longer receiving cysteamine.

Experiments with unlabeled cystine verified these results.

Table 3. Cystine loss from normal and cystinotic leukocytes loaded with unlabeled cystine dimethyl ester

Time of incubation, min	Half-cystine, nmol/mg cell protein			
	Normal control 1	Cystinotic patient 1*	Normal control 2	Cystinotic patient 2†
0	11.2	34.5	38.4	9.6
15	8.2	32.0	34.3	10.7
30	5.7	33.4	29.8	10.2
60	2.5	30.2	20.9	10.1
$t_{1/2}$, min	27	366	67	∞

Cystine was measured by the competitive binding assay (20).
* Sample was obtained 5 hr after a dose of oral cysteamine.
† Sample was from a different patient who had not received cysteamine for more than 24 hr; under these conditions, no cysteamine effect is observed on endogenous leukocyte cystine content.

Normal and cystinotic leukocytes were loaded with nonradioactive cystine dimethyl ester, washed, and incubated in Hanks' solution, and cystine remaining in the cells was assayed by using the cystine-binding protein assay. Whether loaded to relatively high or low cystine levels, and whether or not the cystinotic sample came from a patient whose endogenous leukocyte cystine had been lowered by cysteamine, the normal cells lost substantial amounts of cystine, whereas loss from cystinotic cells, in both rate and absolute amount, was much less (Table 3).

Preliminary results suggest that cultured fibroblasts, like uncultured leukocytes, can be loaded with cystine by using cystine dimethyl ester and that cystinotic fibroblasts demonstrate a corresponding defect in cystine clearance (Fig. 4). Cystinotic and normal fibroblasts were cultivated at 37°C in Eagle's min-

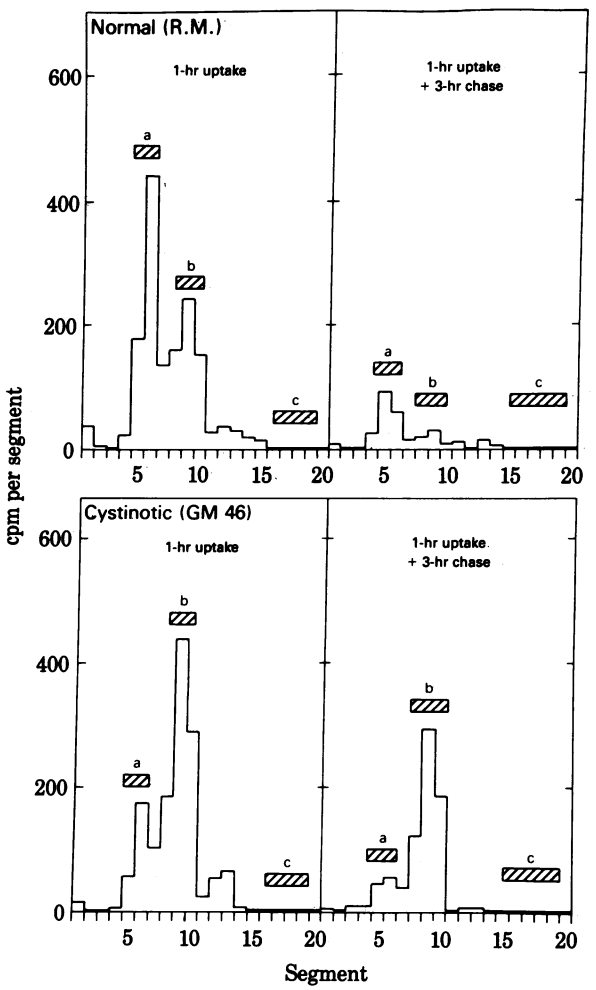

FIG. 4. Cystine clearance in cultured normal and cystinotic fibroblasts. Duplicate cell cultures of normal and cystinotic fibroblasts in 75-cm^2 flasks were incubated for 1 hr at 37°C in 10 ml of cystine-free Eagle's medium containing 0.5 mM [^{35}S]cystine dimethyl ester (50 μCi per flask). One pair of normal and cystinotic cells was then washed three times with phosphate-buffered saline and detached by brief exposure to 0.25% buffered trypsin, and the cells were washed three more times by centrifugation and resuspension in 4 ml of cold phosphate-buffered saline. The final cell pellets were sonicated in 0.5 ml of 10 mM MalNEt (pH 7.0), treated with sulfosalicylic acid (final concentration, 4%), and centrifuged. The remaining cultures of the pair of strains were washed three times with 10 ml of complete Eagle's medium and incubated at 37°C for 3 hr in 10 ml of complete medium containing cystine, after which the cells were processed as above. Aliquots of the resultant protein-free cell extracts were subjected to electrophoretic analysis. Hatched segments: a, cysteine-MalNEt; b, cystine; c, cystine dimethyl ester.

imal essential medium containing 10% fetal calf serum, nonessential amino acids, neomycin, and glutamine. Radioactive cystine dimethyl ester (0.5 mM) was added to the medium for 1 hr so the cystinotic and normal fibroblasts were hyperloaded. There was decreased clearance of radioactive cystine from the cystinotic compared to normal fibroblasts, absolutely and in comparison with radioactive cysteine-MalNEt. After 3 hr of clearance, radioactivity in cystine was almost eliminated from the normal, but not the cystinotic, fibroblasts (Fig. 4).

DISCUSSION

Normal and cystinotic leukocytes can be heavily loaded with intracellular cystine by brief exposure to appropriate concentrations of cystine dimethyl ester; the loading is unaccompanied by toxicity as judged by measurement of other intracellular amino acid concentrations and trypan blue exclusion. Such "hyperloaded" leukocytes contain lucent, sometimes distended, lysosomes, presumably due to cystine accumulation within these organelles, consistent with the demonstration that cystine in leukocytes loaded with the dimethyl ester is concentrated within the lysosome-rich cell fraction.

Clearance of radioactive or nonradioactive cystine from hyperloaded cystinotic leukocytes was markedly and consistently retarded in comparison to normal, whether the cystinotic cells had a grossly increased initial cystine pool (untreated cystinotic patients) or a slightly increased initial cystine content (patients receiving oral cysteamine therapy). These observations provide strong evidence that cystinotic lysosomes *in situ* have a defective cystine clearance mechanism.

There is no convincing evidence for cystine reduction to cysteine within leukocyte lysosomes (21). Intralysosomal cystine might traverse the lysosomal membrane as such and be subsequently reduced in the cytoplasm by glutathione or other reducing compounds. Thus, the lower ratios of radioactive cysteine-MalNEt to cystine in loaded cystinotic cells compared to loaded normal cells are also consistent with a defect in clearance of cystine from cystinotic lysosomes.

Preliminary results in cystinotic fibroblasts are consistent with those observed in leukocytes, suggesting that both types of cells will be useful in further studies to define the etiology of the clearance defect, especially in investigations involving isolated lysosomes (12–14, 20, 22, §, ¶).

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