Cystine Transport Is Defective in Isolated Leukocyte Lysosomes from Patients with Cystinosis

Abstract. *The activity of a cystine transport system in lysosomes prepared from the leukocytes of patients with cystinosis was found to be deficient. In normal subjects, this system was resistant to* N-*ethylmaleimide and demonstrated saturation kinetics. Lysosomes from individuals heterozygous for cystinosis demonstrated a reduced maximum velocity for cystine egress from lysosomes. The rate of cystine escape from normal lysosomes was enhanced by adenosine triphosphate. The availability of normal and mutant lysosomes provides a means of investigating mechanisms of amino acid transport across lysosomal membranes.*

Most lysosomal storage diseases result from defective intralysosomal catabolism of macromolecules, whereas transport disorders appear to involve abnormal plasma membrane carrier systems (*1*). We now report that the malfunction of a carrier-mediated lysosomal system for cystine transport results in the lethal storage disease nephropathic cystinosis (*2*).

Nonprotein cystine accumulates to excessive amounts within the lysosomes of cystinotic fibroblasts, leukocytes, and certain other cells (*3, 4*). For experimental purposes, normal lysosomes can be loaded with cystine to cystinotic levels by exposure to cystine dimethyl ester. Amino acid methyl esters readily cross membranes and are hydrolyzed intralysosomally to more poorly diffusible, free amino acids which then accumulate (*5, 6*).

When whole leukocytes (*7*) and lysosome-rich granular fractions (*8, 9*) from cystinotic patients were loaded by exposure to cystine dimethyl ester, cystine elimination was grossly retarded compared with preparations from normal subjects (*9, 10*). The studies we report here suggest that lysosomal cystine transport in normal leukocytes is saturable and is stimulated by adenosine triphosphate (ATP), consistent with a carrier-mediated process; that lysosomes from the leukocytes of cystinotic patients exhibit virtually no transport of cystine, consistent with deficient carrier activity; and that lysosomes from leukocytes of individuals heterozygous for cystinosis display approximately half the normal maximum rates of cystine transport, suggesting a gene dosage effect. Metabolic interconversions between cystine and cysteine do not account for deficient cystine egress from cystinotic lysosomes.

Leukocytes, prepared by dextran sedimentation and hypotonic lysis of erythrocytes, were exposed to cystine dimethyl ester (Sigma) or [^{35}S]cystine dimethyl ester (209 Ci/mole), prepared from [^{35}S]cystine (Amersham) (*8*), for 30 minutes at 37°C in Hanks balanced salt solution (Ca^{2+}- and Mg^{2+}- free) containing 10 m*M* sodium phosphate, *p*H 7.0. After brief sonication, the cells were centrifuged at 85,000*g* and the lysosome-rich fractions were isolated (*8*) and incubated at 37°C in 0.25*M* sucrose with 10 m*M* Hepes, *p*H 7.0, without additives except as indicated. Portions were then removed and centrifuged at 8500*g* for 10 minutes, and pellets and supernatants were analyzed for cystine by a binding protein assay (*12*) or for [^{35}S]cystine and [^{35}S]cysteine NEM (*N*-ethylmaleimide; Pierce Chemical Co.) by high-voltage paper electrophoresis (*8*).

As a measure of lysosomal rupture, hexosaminidase was assayed in the supernatant of each centrifuged portion and compared with the total activity of each portion in the presence of 0.1 percent Triton X-100. For each assay, 100 μl of protein suspension was incubated at 37°C for 3 minutes with 200 μl of 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (1.2 m*M*) in either 0.1*M* sodium acetate, *p*H 5.0 (*13*), or in 13 m*M* citric acid with 20 m*M* sodium phosphate, *p*H 4.4, containing 0.25*M* sucrose (*14*). One unit of hexosaminidase hydrolyzed 1 nmole of substrate per minute at 37°C in citrate.

We found that 1 m*M* NEM had no effect on lysosomal integrity as gauged by the percentage of hexosaminidase activity released during incubation of cystine-loaded granular fractions at 37°C for 45 minutes with or without this reagent (data not shown).

In order to assess whether cystine leaves lysosomes as cystine or only after reduction to cysteine, we measured [^{35}S]cystine and [^{35}S]cysteine in washed lysosomes and supernatants in the presence or absence of NEM throughout the incubation (*15*). Data from a representative experiment are shown in Table 1. The total radioactivity from cysteine and cystine in the pellet plus the supernatant remained constant throughout the incubation period and was not affected by the presence of 1 m*M* NEM. Furthermore, the rate of escape of cystine from the loaded lysosomes was unchanged by the addition of 1 m*M* NEM during incubation.

In the presence of NEM the cystine lost from the lysosomes was fully accounted for by the cystine appearing in the efflux medium; this is incompatible with the view that cystine escape involves a preliminary reduction to cysteine, in which case cysteine would be trapped by NEM and the amount of cystine lost from the lysosomes would exceed that recovered as cystine outside. In the absence of NEM, the amount of cystine appearing outside the lysosomes exceeded the amount of cystine that escaped from the lysosomes, compatible with extralysosomal oxidation of effluxed cysteine.

For subsequent experiments we used sucrose-Hepes containing 1 m*M* NEM and the granular fractions prepared from leukocytes loaded with unlabeled cystine dimethyl ester. The appearance of cystine outside the lysosomes and the disappearance of cystine from within the lysosomes were monitored with the cystine binding assay. The total amount of cystine recovered remained constant as the amount inside the lysosomes decreased and the amount outside increased (Fig. 1). When the amount of cystine outside the lysosomes was corrected for the cystine present because of rupture of a small

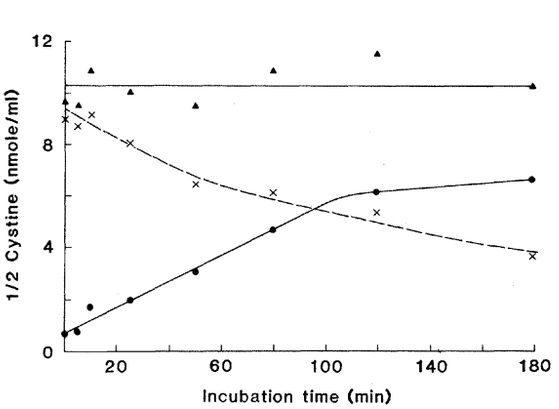

Fig. 1. Recovery of cystine from the soluble and granular fractions after incubation of loaded leukocyte granular fractions for 0 to 180 minutes. Normal leukocytes were loaded with 1 m*M* cystine dimethyl ester and the granular fractions were incubated at 37°C. Portions were removed at various times and centrifuged at 8500*g* for 10 minutes; the cystine in each pellet (×) and supernatant (●) was determined, as well as the total cystine recovered (▲).

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percentage of lysosomes (as indicated by hexosaminidase release), the appearance of cystine in the supernatant increased in approximate proportion to time through at least 40 minutes of incubation. This was observed in granular fractions from leukocytes loaded with cystine by using 0.1 to 1.0 m*M* dimethyl ester.

Calculations of the velocity of cystine appearance outside the lysosomes were based on samples obtained between 0 and 30 minutes of incubation, at different levels of lysosomal loading with cystine. Leukocytes from normal blood were exposed to 0.1 to 2.0 m*M* cystine dimethyl ester and their lysosomes incubated at 37°C in sucrose, Hepes, and 1 m*M* NEM. The velocity of cystine escape at each level of cystine loading was measured (Fig. 2). The velocity increased with progressively higher concentrations of intralysosomal cystine up to about 0.5 nmole of 1/2 cystine per unit of hexosaminidase, and then leveled off or increased more slowly. From double-reciprocal plots, we calculated that the maximum velocity (V_{max}) in normal lysosomes was approximately 23 pmole of 1/2 cystine per minute per unit of hexosaminidase.

Velocity determinations at different levels of cystine loading in lysosomes from obligate heterozygotes for cystinosis revealed a similar plateau phenomenon. The V_{max} in heterozygotes was reduced to approximately one-half normal (Fig. 2).

When leukocyte granular fractions from four cystinotic patients were each loaded to a single cystine concentration by exposure to 0 to 0.25 m*M* cystine dimethyl ester, the velocity for cystine egress was approximately zero (Fig. 2), whether or not the patients were receiving oral cysteamine (*16*). In patients X_1 and X_3, zero time cystine was five to ten times the endogenous cystine content, eliminating the possibility that reduced cystine efflux in cystinosis is solely an artifact of high endogenous cystine concentrations.

In normal but not in cystinotic leukocyte lysosomes, the rate of cystine egress was stimulated approximately 2.5-fold by 1.25 m*M* ATP (in the presence of 1.25 m*M* $MgCl_2$, 94 m*M* KCl, 82 m*M* sucrose, and 10 m*M* Hepes, *p*H 7.0). Normal leukocytes incubated in Hanks balanced salt solution with 10 m*M* NaF at 37°C for 30 minutes prior to loading with methyl ester showed an approximately 80 percent reduction in cellular ATP (*17*), and cystine egress was inhibited to 24 percent of control (*18, 19*).

The search for a defective cystine transport system in cystinosis has long been hampered by the grossly unequal initial cystine content of normal and cystinotic lysosomes. The use of cystine dimethyl ester to load lysosomes allows comparison of normal and cystinotic rates of cystine egress from comparably loaded isolated leukocyte granular fractions.

Cystine egress from normal leukocyte lysosomes is primarily carrier-mediated rather than diffusional, since the velocity of egress is saturable as a function of cystine loading (Fig. 2), and cystine transport is accompanied by counter-transport (*19*), a characteristic of carrier-mediated, not diffusional, systems. Lysosomal cystine transport, and counter-transport, is grossly deficient in cystinosis. Heterozygotes for cystinosis display approximately half-normal maximal rates of cystine transport from their lysosomes (Fig. 2). In addition, normal (but not cystinotic) cystine egress is ATP-

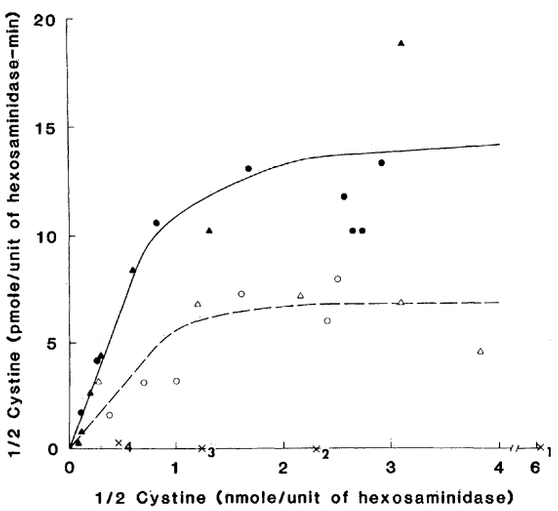

Fig. 2. Velocity of cystine egress from granular fractions isolated from leukocytes of normal subjects, cystinotic patients, and subjects heterozygous for cystinosis. The abscissa shows the loading achieved in the granular fraction at the initiation of incubation in cystine per unit of hexosaminidase activity; the ordinate indicates initial velocity expressed as pmole of 1/2 cystine per minute appearing in the supernatant over the first 30 minutes per unit of initial hexosaminidase activity, corrected for lysosomal rupture as judged by hexosaminidase release. Leukocytes from two normal subjects (●, ▲) and two obligate heterozygotes for cystinosis (○, △) were loaded with 0.1 to 2.0 m*M* unlabeled cystine dimethyl ester. Granular fractions were prepared and velocities determined at each level of loading. Leukocytes from four different cystinosis patients (X_1 to X_4) were loaded to a single cystine level and the velocity of cystine egress from isolated granular fractions was determined. Patients X_2 and X_3 were not taking cysteamine; blood from X_1 and X_4 was taken 5 hours after a cysteamine dose.

Table 1. Recovery of [^{35}S]cystine and [^{35}S]cysteine inside and outside lysosomes loaded with [^{35}S]cystine dimethyl ester and incubated in sucrose-Hepes, *p*H 7.0, with or without 1 m*M* *N*-ethylmaleimide (NEM). Normal leukocytes were exposed to 0.25 m*M* [^{35}S]cystine dimethyl ester. This loaded the lysosomes with cystine and cysteine, the latter presumably arising from cytoplasmic reduction. The granular fraction was divided into equal portions and suspended in 0.25*M* sucrose containing 10 m*M* Hepes, *p*H 7.0, with or without 1 m*M* NEM. At 0, 15, 30, and 45 minutes, 1-ml portions were centrifuged for 10 minutes at 8500*g*; 400 μl of each supernatant was added to 40 μl of 0.1*M* NEM in 0.1*M* sodium phosphate, *p*H 7.0. Each pellet was resuspended in 500 μl of 10 m*M* NEM, freeze-thawed three times in Dry-Ice acetone, and after several minutes was acidified with 50 μl of 40 percent sulfosalicylic acid and centrifuged. High-voltage paper electrophoresis was performed on 20-μl portions of each supernatant and pellet extract, and the radioactivity in cystine and cysteine-NEM was determined. Radioactivity is expressed as counts per minute in spots comigrating with authentic cystine or cysteine-NEM. Soluble hexosaminidase activity increased from 3 to 9 percent after 45 minutes at 37°C, whether or not NEM was present.

Radioactivity inside lysosomes			Radioactivity outside lysosomes		
Time (min)	[^{35}S]-Cystine	[^{35}S]-Cysteine	Time (min)	[^{35}S]-Cystine	[^{35}S]-Cysteine
		Without NEM in the medium			
0	46.0	152.8	0	15.0	8.8
15	33.5	67.5	15	59.8	57.0
30	28.3	38.0	30	79.0	70.5
45	22.3	24.5	45	111.5	64.8
		With NEM in the medium (10^3 count/min)			
0	43.8	157.0	0	10.0	14.3
15	33.0	117.3	15	24.8	52.0
30	26.3	86.8	30	32.8	77.5
45	23.5	71.5	45	37.5	93.5

dependent. Cystine egress is not influenced by 1 m*M* NEM and proceeds without intermediary conversion to cysteine (Table 1). Since methionine, tryptophan, and leucine egress are not substantially altered in cystinotic lysosomes (*8, 9*), these amino acids probably do not share a lysosomal transport mechanism with cystine.

The demonstration of a natural cystine transport system in lysosomes, and the availability of mutant lysosomes wholly and partially deficient in such transport, should permit elucidation of fundamental information on this aspect of lysosomal function. Furthermore, the possibility is suggested that other lysosomal transport systems may exist and that their dysfunction could play a role in certain other lysosomal storage diseases.

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19. Data indicate that normal lysosomes display countertransport of cystine when intralysosomal cystine approaches saturation concentrations (W. Gahl, F. Tietze, N. Bashan, I. Bernardini, J. D. Schulman, in preparation).
20. Presented in part at the Society for Pediatric Research, Washington, D.C., May, 1982 and the International Congress on Glutathione, Karolinska Institutet, Stockholm, May 1982.

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Transmethylation of Phosphatidylethanolamine: An Initial Event in Embryonic Chicken Lens Fiber Cell Differentiation

Abstract. *Agents that induce differentiation of lens epithelial cells into lens fiber cells in vitro transiently stimulate the transmethylation of phosphatidylethanolamine. Inhibition of transmethylation by 3-deazaadenosine results in a corresponding inhibition of the cell elongation that characterizes lens fiber formation, suggesting that phospholipid methylation plays an essential role in the differentiation of these cells.*

Explants of lens epithelium from chicken embryos in early development differentiate to form lens fibers when cultured in the presence of fetal calf serum (*1, 2*), vitreous humor (*3*), or insulin (*4*). As the cells differentiate in vitro they undergo the morphological, ultrastructural, and biochemical changes that characterize lens fiber cell formation in vivo (*5, 6*). The cells elongate (*1–4*), cease dividing (*7, 8*), and accumulate messenger RNA for the lens-specific protein δ-crystallin (*9, 10*), thus becoming highly specialized for δ-crystallin synthesis (*2, 10, 11*). Although the initial events leading to lens fiber cell formation are not known, it is likely that a cell surface receptor is involved, since both insulin and a 60,000-dalton protein present in the vitreous humor (*3*) are capable of producing lens fiber formation in vitro. Recently it was shown that a transient stimulation of the transmethylation of phosphatidylethanolamine to phosphatidylcholine is an early event associated with the binding of a number of proteins and peptides to their respective cell surface receptors (*12*). Phospholipid methylation is thought to initiate a chain of events leading to a cellular response, such as secretion in mast cells, chemotaxis in leukocytes, and neuritic outgrowth in cultured superior cervical ganglia (*12*). The present study was undertaken to determine whether the transmethylation of phosphatidylethanolamine to phosphatidylcholine is involved in the initiation of lens fiber cell differentiation.

Square explants 0.7 mm on a side and containing approximately 10^4 cells were cut from the central region of the lens epithelium of 6-day-old embryonic chickens (*2*). A differential interference contrast microscope was used to measure cell length in living explants 1 and 2.5 hours after stimulation (*13*). For each

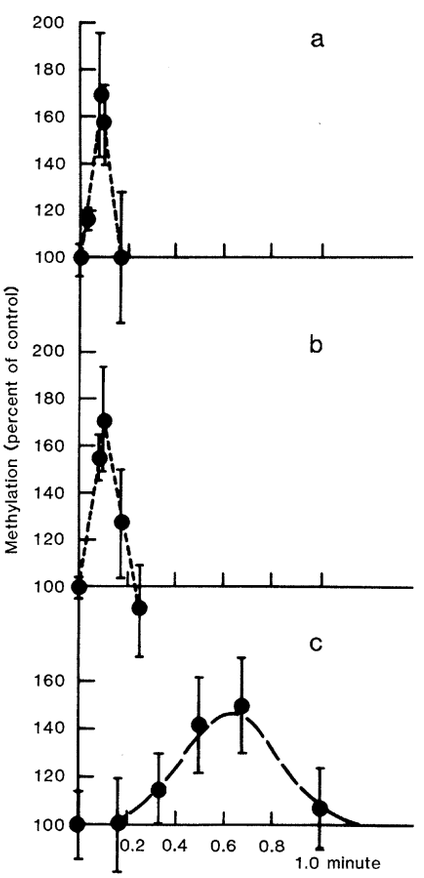

Fig. 1. Incorporation of ^3H from L-[methyl-^3H]methionine into phosphatidylcholine as a function of time after the addition of (a) fetal calf serum, (b) embryonic chicken vitreous humor, or (c) bovine insulin. Each data point is the mean ± standard error for 6 to 30 measurements.

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