Pediat. Res. *10:* 637–641 (1976)

L-Cystine
cystinosis
fibroblasts
lysosome
plasma membrane

Transport of L-Cystine by Cultivated Skin Fibroblasts of Normal Subjects and Patients with Cystinosis

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Extract

Uptake of L-cystine at the plasma membrane of fibroblasts derived from normal and cystinotic subjects was studied. L-Cystine accumulation after a 20-min period was increased in cystinotic fibroblasts incubated in 0.08 mM L-cystine. This effect appeared to be concentration-dependent since accumulation after 20 min at 0.004 mM concentration was decreased in cystinotic cells. Kinetic data suggested that at least two nondiffusional saturable processes with widely different substrate affinities mediate initial L-cystine uptake in skin fibroblasts. In addition, the transport process with high affinity for L-cystine may itself be a two-component system, as suggested by (*1*) additive inhibtory effect of other neutral amino acids, and (*2*) preincubation studies in which preincubation with cystathionine enhanced subsequent L-cystine uptake, whereas preincubation with other neutral amino acids depressed subsequent uptake. Affinity constants and maximal velocities of initial uptake did not appear to be altered in cells derived from patients with cystinosis. After 60-sec incubation with L-[^{35}S]cystine, cystinotic cells retained more label as cystine than did normal cells at each concentration studied.

These data indicate that initial L-cystine uptake in fibroblasts of patients with cystinosis proceeds at a normal rate by means of all transport systems currently shown to be present in normal cells.

Speculation

L-Cystine may accumulate in cells of patients with cystinosis because of an alteration in a transport system for L-cystine which is, as yet, undescribed. Alternatively, L-cystine accumulation may occur because of a transport defect at the lysosomal membrane which is not expressed at the plasma membrane.

Nephropathic cystinosis is a recessively inherited disorder characterized by lysosomal accumulation of L-cystine in cells of affected individuals (17). The nature of the biochemical defect in this disease has been the subject of much investigation. Since no enzymatic defect which might lead to L-cystine accumulation has been demonstrated (8, 20, 21), a number of investigators have postulated that abnormal transport of L-cystine or L-cysteine at the plasma or lysosomal membrane may be responsible for the observed accumulation (17). Schneider *et al.* (15) demonstrated an increased uptake of L-cysteine in leukocytes of cystinotic subjects. In these studies, a rapid conversion of the labeled compound to L-cystine was noted, suggesting that the increased uptake may be a compensatory mechanism for maintaining the physiologic concentration of intracellular amino acid rather than a defect in transport at the plasma membrane. Schulman *et al.* (16) studied uptake of L-cystine in leukocytes growing in continuous culture from patients with cystinosis. The accumulation of L-cystine in cells of affected subjects was similar to controls over a 45-min interval. States *et al.* (18) investigated the uptake of exogenous L-cystine in fibroblasts of cystinotic and normal individuals over a concentration range of 0.05–0.125 mM. They concluded that the apparent K_m for L-cystine uptake was similar in both cell types, although the cystinotic cells demonstrated a higher maximum velocity of entry. A decreased efflux of L-cystine from cystinotic cells was also noted. The purpose of the present investigation is to further clarify the mechanism of L-cystine uptake at the plasma membrane of fibroblasts derived from normal and cystinotic subjects.

MATERIALS AND METHODS

FIBROBLAST CULTURE

Skin biopsies were obtained with informed consent from three individuals with cystinosis and from six control subjects. Fibroblasts were cultivated in Eagle's minimal essential medium (22) supplemented with 15% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/ml Fungizone as described previously (13). All studies were performed on cells between the 6th and 18th passages. When confluent, cells were harvested using 0.25% trypsin in Hank's balanced salt solution.

DETERMINATION OF INTRACELLULAR CYSTINE

This was done according to the method of States and Segal (19).

TRANSPORT STUDIES

Confluent fibroblasts were subcultured onto 20 12-mm no. 1 glass coverslips in Petri dishes for transport studies. The cultures were incubated at 37° in an atmosphere of 5% CO_2 and 95% air and were fed with fresh medium at 2- to 3-day intervals and on the day before transport studies. In studies of transport parameters in different cell strains, cells of the same passage number were compared; cells were subcultured on the same day and were handled together thereafter in all experimental manipulations. On the day of transport studies, growth medium was removed by aspiration and the coverslips were washed twice with phosphate-buffered saline containing $CaCl_2$, 100 mg/liter; $MgCl_2 \cdot 6H_2O$, 100 mg/liter; and glucose 0.1% (PBS-G). Fresh PBS-G was then added and the attached cells were incubated at 37° for 30 min before transport studies were initiated (4).

Substrate solutions for transport studies consisted of amino acids in the indicated concentrations in PBS-G at 37°. L-[^{35}S]Cystine, 250 mCi/mmol, was obtained from Amersham/Searle (23). The radiochemical was subjected to high voltage paper electrophoresis at pH 1.9, 4,000 V (50–75 ma), for 45 min; radioactivity migrated as a single component with the same mobility as L-cystine; no radioactive impurities were detected. Coverslips at confluency were blotted dry and then dipped in the incubation medium with continuous agitation. At the end of the incubation period, the

coverslip was rinsed by rapidly dipping through three containers of normal saline. The edge of the coverslip was then touched to filter paper and the coverslip was dropped into a scintillation vial, to which was added 10 ml 1:1 toluene-methyl-Cellosolve with 0.4% 2,5-diphenyloxazole (PPO) and 0.005% *p*-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) (24). Radioactivity of the coverslips and of the substrate solution after incubation were measured in a liquid scintillation counter. In each experiment, three coverslips were dipped briefly in substrate solution and then rinsed and counted as above. These coverslips served as the "zero time" controls.

Distribution ratios (defined as the ratio of concentration of amino acid in intracellular fluid to concentration of amino acid in the substrate solution) were used as a measure of intracellular amino acid accumulation. Intracellular water was measured as space accessible to [^{14}C]urea but not to [^{14}C]inulin. Replicate coverslips from the same Petri dish were exposed to [^{14}C]urea (2–10 mCi/mmol (25)) or [^{14}C]inulin (inulin-[^{14}C]carboxyl; 1–3 mCi/g (25)) for 30 min. The coverslips were removed, touched to filter paper, and counted for radioactivity. Protein was measured by the method of Lowry *et al.* (12) applied directly to cells on coverslips which had been well rinsed to remove growth medium. At least three coverslips from each dish were used for protein determination; variation in protein content per coverslip in each Petri dish was less than 5%. As has been previously reported (1, 4), intracellular water space was directly proportional to protein content per coverslip. Because data obtained from Arrhenius plots indicated that nondiffusional processes were responsible for L-cystine uptake at both high and low L-cystine concentrations (see *Results*), uptake was not corrected for diffusion.

Accumulation of L-[^{35}S]cystine by cystinotic and normal fibroblasts was determined at substrate concentrations of 0.004, 0.010, 0.020, 0.040, and 0.080 mM over a 20-min incubation period.

Inhibition of L-cystine uptake by amino acids was performed at 60-sec incubation periods utilizing a wide variety of amino acids at 1 mM concentration in the substrate solution. All amino acids were obtained from Sigma Chemical Company (26), and were Sigma grade. Serine, cystathionine, and homocystine contained no detectable cystine by thin layer chromatography.

DETERMINATION OF INTRACELLULAR LABEL AFTER 60-SEC INCUBATION

Forty-eight hours after subculture, fibroblasts were washed free of medium with PBS-G. PBS-G containing L-[^{35}S]cystine at concentrations of 0.004–0.150 mM was then added to the monolayers. The cells were incubated at 37° for 60 sec, after which the substrate solution was poured off and the cells were immersed in ice and rapidly cooled to a temperature of 4°. The cells were washed three times in ice-cold saline and then removed from the flasks by scraping with a rubber policeman in the cold. The cell suspensions were washed with ice-cold saline two additional times to remove any residual traces of substrate solution. PBS-G containing 20 mM *N*-ethylmaleimide (NEM; Sigma), 0.5 ml, was then added to the cell pellet and the cells were freeze-thawed five times in Dry Ice. Three percent sulfosalicylic acid, 0.5 ml, was then added to the solution which was centrifuged at 20,000 rpm in a 40 rotor of a Spinco model L ultracentrifuge for 30 min. An aliquot of the clear supernatant solution was subjected to high voltage paper electrophoresis at 4000 V, pH 1.9 (50–75 ma), for 45 min for separation of amino acids. The paper was cut into 1-cm strips and radioactivity was located by counting in a liquid scintillation counter. Standard solutions of L-cystine, L-cysteine-NEM, and glutathione-NEM were run simultaneously with the sample for determination of the positions of these known substances. Glutathione-NEM and L-cysteine-NEM were not well separated from one another by this procedure; both compounds, however, were readily distinguished from L-cystine.

RESULTS

CYSTINE CONTENT OF CYSTINOTIC AND NORMAL FIBROBLASTS

The mean intracellular content of free cyst(e)ine in skin fibroblasts of three patients with cystinosis was 5.16 nmol/mg protein (range: 4.70–5.39 nmol/mg protein), whereas cyst(e)ine content in cells of five control individuals was less than 1.0 nmol/mg protein. The method as applied is specific for cyst(e)ine.

TIME COURSE OF [^{35}S]CYSTINE UPTAKE BY CONTROL AND CYSTINOTIC FIBROBLASTS

The results of timed accumulation studies are illustrated in Figures 1 and 2. Two cystinotic cell lines were compared with two normal cell lines in paired experiments of L-cystine accumulation at 0.010, 0.020, 0.040, and 0.080 mM concentrations. At 0.010 mM substrate concentration, cystinotic and normal cells appeared to accumulate L-cystine at approximately the same rate. At 0.020, 0.040, and 0.080 mM L-cystine concentrations, cystinotic cells appeared to accumulate more L-cystine than did normal cells over the time period studied, as has previously been reported (18). A representative experiment is illustrated in Figure 1. In contrast, when two cystinotic cell lines were compared with three normal cell lines in a total of nine paired experiments of L-cystine accumulation at 0.004 mM concentration, cystinotic fibroblasts accumulated less L-cystine than did normal cells (mean uptake of cystinotic cells was 74% ± 16% of normal at 20 min, $p < 0.02$). A representative experiment is shown in Figure 2. At each substrate concentration, uptake was maximal during the first 2 min and then rapidly plateaued. Constant distribution ratios were not obtained by 30 min. Therefore, all studies of the kinetics of cystine uptake were performed at an incubation time of 60 sec in order to study initial rates of uptake.

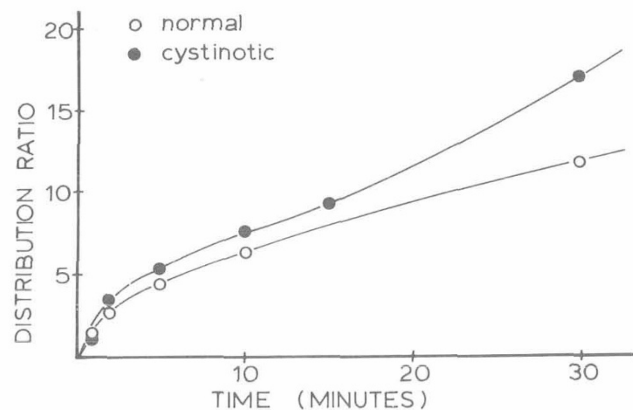

Fig. 1. Accumulation of 0.080 mM L-[^{35}S]cystine in cystinotic and normal fibroblasts.

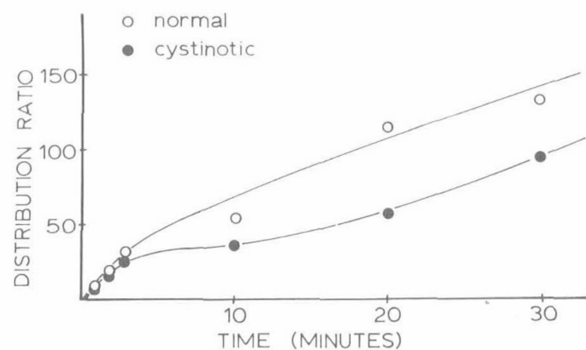

Fig. 2. Accumulation of 0.004 mM L-[^{35}S]cystine in cystinotic and normal fibroblasts.

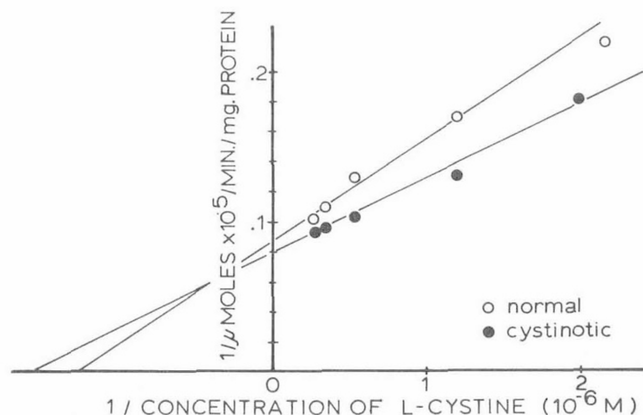

Fig. 3. Kinetics of L-cystine accumulation at low substrate concentration. The reciprocal of velocity of accumulation is plotted versus the reciprocal of initial substrate concentration for determination of the K_m and V_{max} of transport by means of the high affinity system.

INITIAL RATE KINETICS

The relationship of initial velocity of uptake to concentration of L-cystine was investigated over a concentration range of 0.3×10^{-6}–0.8×10^{-3} M in normal and cystinotic cells. In both normal and cystinotic cells, two saturable processes of L-cystine accumulation with widely different substrate affinities were demonstrated. The first process (high affinity system) demonstrated saturation at approximately 0.004 mM L-cystine concentration. When the reciprocal of velocity of uptake was plotted *vs.* the reciprocal of substrate concentration according to the method of Lineweaver and Burk (11), the apparent K_m was calculated to be 0.00078 mM in the normal cell line (range: 0.00040–0.0012 in five experiments); the V_{max} was 38 μmol/liter cell water·min (range: 28–56 μmol/liter cell water·min in five experiments). In the affected cell line, the K_m was 0.00061 mM (range: 0.00050–0.0011 in five experiments) and the V_{max} was 41 μmol/liter cell water·min (range: 30–61 in five experiments) (Fig. 3). The second process (low affinity system) appeared saturable at very high substrate concentrations. The apparent K_m was 0.37 mM L-cystine (range: 0.30–0.44 mM in three experiments) and the V_{max} was 0.74 mmol/liter cell water·min (range: 0.52–0.80 in three experiments) in both normal and affected cells (Fig. 4).

EFFECT OF TEMPERATURE ON ACCUMULATION OF L-CYSTINE

In order to determine the contribution of diffusion to L-cystine uptake by means of the high and low affinity transport systems, accumulation at 0.005 mM and 0.5 mM initial substrate concentrations at varying temperatures was investigated. In each instance, uptake velocity varied exponentially with absolute temperature (Fig. 5). The Q_{10} at 0.5 mM was 1.8 and the Q_{10} at 0.005 mM was 2.3 (T° + 10 = 41°). The rate of a diffusional process is directly proportional to absolute temperature whereas the rate of a chemical reaction varies exponentially with temperature. In general, the temperature coefficient (Q_{10} = the ratio of reaction rates at T + 10° and T°C) in free diffusion in an aqueous solution is less than 1.5 (2). Thus, nondiffusional processes appeared to account for accumulation of L-cystine at both low and high substrate concentrations.

INHIBITION STUDIES

Inhibition of transport of L-cystine at an initial substrate concentration of 0.004 mM was studied utilizing a wide variety of amino acids at 1 mM concentration in the substrate solution (Table 1). L-Serine, L-homocystine, and L-cystathionine were found to partially inhibit L-cystine uptake, whereas L-glutamic acid, L-tryptophan, L-lysine, L-leucine, and L-methionine had no effect on the initial rate of L-cystine accumulation in normal or affected cells. Ascorbic acid at 0.57 mM concentration also had no effect on the initial rate of L-cystine accumulation in normal or affected cells. L-Serine and L-homocystine and L-serine and L-cystathionine in combination appeared to exhibit an additive effect (Figs. 6 and 7), suggesting that transport of L-cystine at 0.004 mM concentration may be a two-component system (3).

EFFECT OF PRELOADING ON INITIAL UPTAKE VELOCITY

The effect of high intracellular concentrations of a specific amino acid on initial uptake of L-cystine was investigated. Cells were preincubated with a solution containing a 1 mM concentration of the specific amino acid suspended in PBS-G for 30 min at 37°. Control cells were preincubated in PBS-G. Initial uptake of L-cystine at 0.004 mM concentration was determined. The results are shown in Table 2. Most of the amino acids tested resulted in

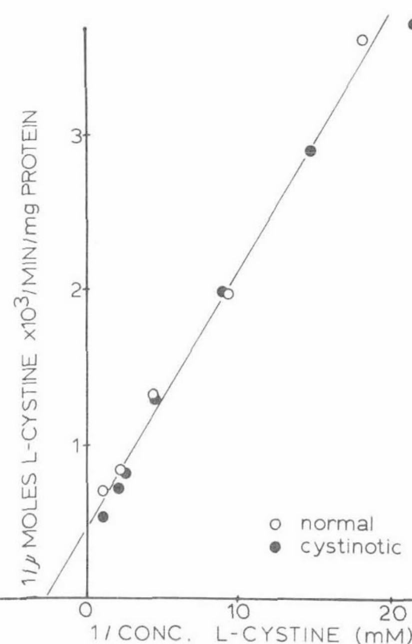

Fig. 4. Kinetics of L-cystine accumulation at high substrate concentration. The K_m and V_{max} of transport by means of the low affinity system are determined as in Fig. 3.

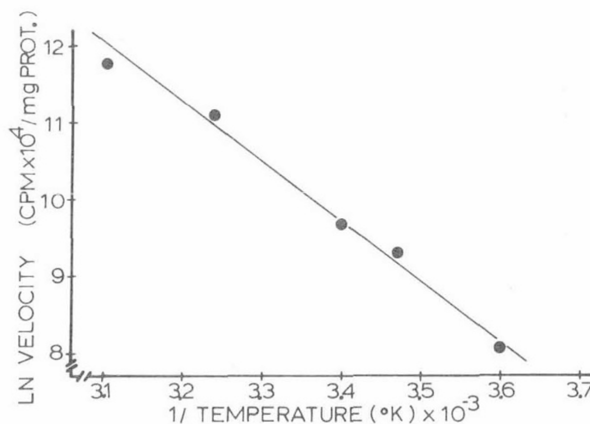

Fig. 5. Variation of velocity of L-cystine accumulation by the high affinity transport system with temperature. The natural logarithm of velocity of uptake is plotted *vs.* the reciprocal of absolute temperature in an Arrhenius plot; a linear relationship by this treatment indicates that the velocity of transport varies exponentially with temperature. A similar relationship was observed when the low affinity system was investigated.

decreased uptake of approximately 50%. In contrast, L-cystathionine enhanced uptake approximately 200%, also suggesting the possibility of two transport systems with differing mechanisms of transport at this substrate concentration (6, 10).

INTRACELLULAR FATE OF L-[^{35}S]CYSTINE

The fate of intracellular label after 60-sec incubation at varying substrate concentrations is shown in Table 3. At each concentration tested, cystinotic cells retained more label as cystine. In addition, the degree of label remaining as cystine was unaffected by the substrate concentration.

DISCUSSION

These data indicate that uptake of L-cystine in both normal and cystinotic fibroblasts is a complex process involving more than a single nondiffusional transport system. The kinetic data suggest that at least two saturable processes with widely different substrate affinities mediate L-cystine uptake in skin fibroblasts. In addition, the transport process with high affinity for L-cystine may in itself be a two-component system, as suggested by the additive inhibitory effects of other neutral amino acids (3). This hypothesis is strengthened by the preincubation experiments. Preincubation with L-cystathionine enhances subsequent L-cystine uptake, whereas preincubation with other neutral amino acids depresses subsequent uptake, suggesting the possibility of two transport systems with differing mechanisms of transport at this substrate concentration (6, 10). Affinity constants and maximal velocities of initial uptake do not appear to be altered in cells derived from patients with cystinosis.

These findings are in sharp contrast to the work of States *et al.* (18), who reported an apparent K_m of L-cystine uptake of 0.043 mM in both normal and cystinotic cells and an increased V_{max} of uptake in cells derived from subjects with cystinosis. These differences may be explained when the methodology is reviewed. In the studies of States *et al.* (18), all kinetic investigations were performed at incubation periods of 20 min, at which time, according to their data and ours, uptake is no longer linear with time. In addition, both studies indicate that metabolism of substrate has proceeded to a considerable degree by 20 min. There-

Table 1. *Inhibition of 0.004 mM L-cystine accumulation by amino acids in skin fibroblasts*[1]

Inhibitor	Distribution ratio at 60 sec	
	Normal	Cystinotic
None	8.7	9.0
L-Serine	3.9	4.6
L-Cystathionine	3.2	4.0
L-Homocystine	4.1	3.9
Glutathione (oxidized)	7.8	8.0
L-Lysine	9.8	9.1
L-Glutamic acid	8.0	8.8
L-Tryptophan	10.0	9.9
L-Methionine	8.1	8.6
L-Leucine	7.1	8.0
L-Cystine (0.5 mM)	0.93	1.1
Ascorbic acid (0.57 mM)	9.4	9.3

[1] All amino acids listed are at 1 mM concentration unless otherwise specified.

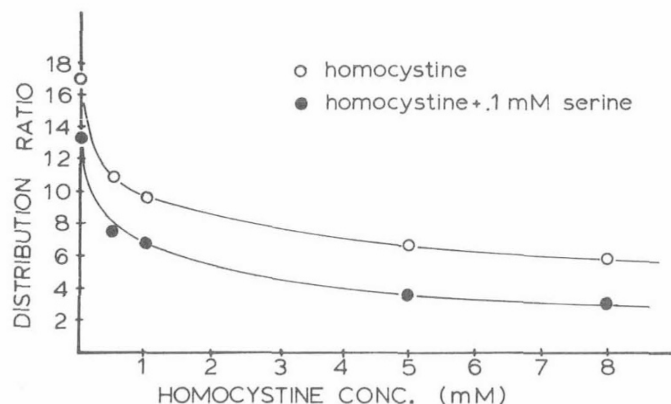

Fig. 6. Inhibition of accumulation of L-cystine at 0.004 mM concentration by homocystine and homocystine plus serine. The *open circles* (O) representL-cystine accumulation in the presence of varying homocystine concentrations; the *closed circles* (●) represent L-cystine accumulation in the presence of varying homocystine concentrations and 0.1 mM L-serine.

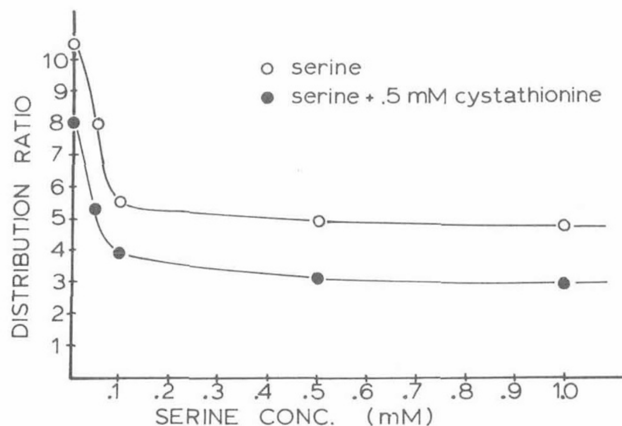

Fig. 7. Inhibition of accumulation of L-cystine at 0.004 mM concentration by serine and cystathionine. The *open circles* (O) represent L-cystine accumulation in the presence of varying serine concentrations; the *closed circles* (●) represent L-cystine accumulation in the presence of varying serine concentrations and 0.5 mM cystathionine.

Table 2. *Effect of preincubation on uptake of 0.004 mM L-cystine by skin fibroblasts*

Preincubation solution	% Uptake at 60 sec	
	Normal	Cystinotic
Phosphate-buffered saline + 0.1% glucose	100	100
Serine, 1 mM	45	40
Homocystine, 1 mM	68	67
Reduced glutathione, 1 mM	75	80
Cystathionine, 1 mM	207	180

Table 3. *Metabolic fate of ingested L-[^{35}S]cystine after 60-sec incubation*

Cell line	Concentration of L-cystine, mM	% Label as L-cystine
Normal	0.004	23
	0.016	13
	0.032	19
	0.080	17
Cystinotic	0.004	56
	0.016	57
	0.032	50
	0.080	55

fore, the kinetic data previously reported cannot be considered to represent initial rates of transport but rather a combination of accumulation and metabolism.

Data presented in the present studies confirm the observation of States *et al.* (18) that L-cystine accumulation after a 20-min period is increased in cystinotic fibroblasts incubated in 0.080 mM L-cystine. This effect appears to be concentration dependent, as indicated by the finding that accumulation of L-cystine after 20 min at 0.004 mM concentration is decreased in cystinotic cells.

Groth and Rosenberg (5) investigated initial rates of accumulation of L-cystine and dibasic amino acids in cultivated fibroblasts derived from normal subjects and from individuals with cystinuria, an inherited disorder of renal and intestinal amino acid transport unreleated to cystinosis. The initial velocity of L-cystine uptake was studied over a range of L-cystine concentration of 0.025–0.50 mM, and an apparent affinity constant of 0.71 mM was calculated. This finding is in good agreement with our "low affinity" transport system. Groth and Rosenberg (5) did not investigate L-cystine uptake at initial substrate concentrations below 0.025 mM and, therefore, the additional concentrative and mediated transport systems described in this study were not observed.

Kroll and Schneider (9) have recently reported a decrease in free cystine content of cystinotic skin fibroblasts grown in tissue culture medium containing 0.57 mM ascorbic acid. Ascorbic acid does not alter initial rates of cystine accumulation at 0.004 mM initial substrate concentration; thus, the mechanism by which ascorbic acid decreases intracellular cystine does not appear to be related to an alteration in cystine transport by means of the high affinity, *i.e.*, low substrate, transport systems.

The finding of more than a single transport system for uptake of L-cystine at varying substrate concentrations is of interest. Groth and Rosenberg (5) reported two energy-dependent, saturable processes for the transport of lysine and arginine in cultured fibroblasts. Hillman *et al.* (7) described three transport systems for transport of glycine in isolated mammalian renal tubules. Oxender and Christensen (14) described two distinct transport systems for uptake of neutral amino acids in ascites tumor cells. Thus, the finding of multiple systems capable of transporting a single biologic substrate is not unique. Our data indicate that L-cystine uptake in fibroblasts of patients with cystinosis proceeds at a normal rate by means of all transport systems currently shown to be present in normal cells. However, a defect in L-cystine transport cannot be totally excluded, as the biochemical basis for L-cystine accumulation in this disorder. A subtle alteration in one of these transport systems may yet be present or an additional, as yet undescribed, system may be absent or altered in cells of affected individuals. Alternatively, a defect in transport at the lysosomal membrane which is not expressed at the plasma membrane may account for accumulation within these organelles.

SUMMARY

Initial rates of accumulation of L-cystine were studied in cultivated skin fibroblasts of normal subjects and patients with cystinosis, a recessively inherited disorder characterized by lysosomal accumulation of L-cystine. Two transport systems were detected which appeared to mediate L-cystine uptake; a saturable low affinity transport system (apparent $K_m < 0.37$ mM L-cystine; apparent $V_{max} = 0.74$ mmol/liter cell water·min), and a high affinity transport system (apparent K_m 0.001 mM; apparent $V_{max} = 38–41$ μmol/liter cell water. min). Inhibition and preincubation studies with other neutral amino acids suggested that the high affinity system may itself be composed of more than a single component. All transport systems appeared to be present and unaltered in cells of patients with cystinosis. After prolonged incubation periods, accumulation of 0.080 mM L-cystine was increased in cystinotic cells; this effect was not observed at 0.004 mM L-cystine concentration. After a 60-sec incubation period, 20% of ingested L-[^{35}S]cystine remained in the oxidized form in normal cells while 50–60% of ingested L-[^{35}S]cystine remained in the oxidized form in cystinotic cells. These studies indicate that transport of L-cystine by currently known systems is not altered in fibroblasts of patients with cystinosis.

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25. New England Nuclear, Boston, Mass.
26. St. Louis, Mo.
27. Dr. H. L. Nadler is the Irene Heinz Given and John LaPorte Given Research Professor of Pediatrics.
28. These studies were supported by grants from The National Institutes of Health HD 00036 and HD 04252, The National Foundation-March of Dimes, The Kroc Foundation, and The Helen Fay Hunter Trust.
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30. Accepted for publication January 14, 1976.

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