JCI The Journal of Clinical Investigation

Uptake and Utilization of Exogenous Cystine by Cystinotic and Normal Fibroblasts

Beatrice States, Dorothy Harris, Stanton Segal

J Clin Invest. 1974;53(4):1003-1016. https://doi.org/10.1172/JCI107637.

Research Article

The uptake of l-[^{35}S]cystine was studied in six cystinotic and six normal fibroblast lines grown for five days either on cover slips or in 32-oz plastic flasks. Cystinotics showed greater uptake than normals. The apparent K_t for cystine entry in both types of cells was 0.043 mM but cystinotic cells showed a higher maximum velocity of entry. A comparison of the fate of l-[^{35}S]cystine incubated for 20 min with monolayers of cells showed 30% and 15% of the intracellular ^{35}S to be l-cystine in cystinotic and normal cells, respectively. The ^{35}S effluxed more slowly from cystinotic than from normal cells after a 20-min preloading with l-[^{35}S]cystine. Identification of ^{35}S compounds in efflux media after 3 min showed 75% of the total ^{35}S was l-cystine with the remainder in cysteine and acidic sulfur metabolites of cystine with no essential difference between cystinotics and normals. In paired experiments, the specific activity of the effluxed l-[^{35}S]cystine after both efflux periods was the same as that entering the cell, thus indicating that the free l-[^{35}S]cystine had not exchanged with the pre-existing pool in the cystinotic cells. During 3 min efflux, the l-cystine pool in normal cells was depleted mainly by loss of free cystine. In cystinotic cells, a new steady state was attained after 21 min of efflux and the intracellular l-[^{35}S]cystine had the […]

Uptake and Utilization of Exogenous Cystine by Cystinotic and Normal Fibroblasts

BEATRICE STATES, DOROTHY HARRIS, and STANTON SEGAL

From the Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia, and the Departments of Pediatrics and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19146

ABSTRACT The uptake of L-[^{35}S]cystine was studied in six cystinotic and six normal fibroblast lines grown for five days either on cover slips or in 32-oz plastic flasks. Cystinotics showed greater uptake than normals. The apparent K_t for cystine entry in both types of cells was 0.043 mM but cystinotic cells showed a higher maximum velocity of entry. A comparison of the fate of L-[^{35}S]cystine incubated for 20 min with monolayers of cells showed 30% and 15% of the intracellular ^{35}S to be L-cystine in cystinotic and normal cells, respectively. The ^{35}S effluxed more slowly from cystinotic than from normal cells after a 20-min preloading with L-[^{35}S]cystine. Identification of ^{35}S compounds in efflux media after 3 min showed 75% of the total ^{35}S was L-cystine with the remainder in cysteine and acidic sulfur metabolites of cystine with no essential difference between cystinotics and normals. In paired experiments, the specific activity of the effluxed L-[^{35}S]cystine after both efflux periods was the same as that entering the cell, thus indicating that the free L-[^{35}S]cystine had not exchanged with the pre-existing pool in the cystinotic cells. During 3 min efflux, the L-cystine pool in normal cells was depleted mainly by loss of free cystine. In cystinotic cells, a new steady state was attained after 21 min of efflux and the intracellular L-[^{35}S]cystine had the same percentage of total radioactivity seen after the initial 20-min uptake. After the rapid efflux of L-[^{35}S]cystine from normals, [^{35}S]cysteine and other labeled cystine metabolites appeared in the efflux media. By the end of a 3-min efflux, cystinotic cells had incorporated more label into reduced glutathione than had normal cells. However, when the new steady state was attained in cystinotics, the amounts of ^{35}S in glutathione were not markedly different in the two types of cells. Approximately 95% of the total label could be accounted for in free sulfur compounds.

The data show an increased uptake and decreased efflux of cystine from cystinotic cells. However, it is not possible to conclude if these differences are due to primary changes in membrane function or to the reflection of metabolic defects without further investigation.

Received for publication 26 December 1972 and in revised form 17 December 1973.

INTRODUCTION

The biochemical basis of nephropathic childhood cystinosis, the recessively inherited lethal disease of children characterized by cellular accumulation of cystine crystals, is enigmatic. The finding that leukocytes and cultured fibroblasts from these patients have markedly elevated levels of intracellular cystine (1) has suggested that these cells are a suitable model system in which to determine the molecular basis of the disorder. Fibroblasts from normals and cystinotics have been monitored for disulfide retention in lysosomes (2) and intracellular concentrations of cysteine and reduced glutathione have been measured (3). Schneider, Bradley, and Seegmiller (4) determined some transport characteristics for the reduced compound, cysteine, in leukocytes from cystinotic patients, but refrained from studies of cystine transport because of the poor uptake of the disulfide by normal (5) as well as cystinotic leukocytes (4). Schulman, et al. (6) on the other hand reported that the uptake of [^{35}S] cystine by continuously cultured leukocytes from afflicted children is normal. The various parameters of transport were not extensively studied in these lymphoid cell cultures and there is no evidence for active transport of cystine by them.

Since cystine storage could result from abnormal entry or exit of cystine, we have examined some parameters of L-[^{35}S]cystine movement into and out of normal and cystinotic fibroblasts. Measurements of uptake have

TABLE I

Cystine Content and ICF Measurements of Cultured Fibroblasts

Donor subjects	Age of subject	Passage no. of cells	ICF	½ L-cystine
	yr		*nl/μg protein*	*μmol/g protein*
Cystinotics				
M. M.	3	4	5.06	7.60
M. S.	8	4	4.02	12.00*
A. M.	1	5	4.23	4.12
J. B.	9	5	4.37	2.00
J. E.	6	6	3.20	8.20
		12	2.82	5.76
T. L.	4	15	2.82	7.41
Normals				
F. G.	2	4, 6	3.02	<0.02‡
S. H.	4	5	3.35	<0.02
V. B.	14	5	3.35	<0.02
B. S.	5	6	3.34	<0.02
T. C.	2	12	2.39	<0.02
S. L.	6	15	2.12	<0.02

* Determinations according to the method of States and Segal (14). All other determinations were based on the amino acid analyzer (see text).

‡ In the normal cells, the cystine peak was too small to calculate; therefore the highest cystine content possible was determined based on the known sensitivity of the assay and the protein content of the sample being measured.

been standardized to correct for differences among cell lines in an experimental approach employing monolayers of actively metabolizing cells rather than cell suspensions. The intracellular fate of cystine has been examined to correlate with the uptake and exit of the amino acid from cells. The results form the basis of this report.

METHODS

L-cystine, cysteine-HCL, *N*-ethylmaleimide (NEM),[1] and GSH were purchased from Calbiochem, San Diego, Calif. L-[^{35}S]cystine (specific activity 100 mCi/mM) was obtained from The Radiochemical Centre, Amersham, Bucks, England. [^{14}C]urea (sp act 3.63 mCi/mM), [^{3}H]mannitol (sp act 3.14 Ci/mM), and Liquifluor were purchased from New England Nuclear, Boston, Mass. Scintisol-Complete was a product of Isolab, Inc., Akron, Ohio. Fetal bovine serum and glutamine were purchased from Flow Laboratories, Inc., Rockville, Md. Penicillin, streptomycin, and Minimum Eagle's Medium (MEM) with Earle salts were obtained from Microbiological Associates, Inc., Bethesda, Md. Trypsin (1:250) was purchased from General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio. Thin-layer cellulose plates were obtained from Eastman Kodak Co., Rochester, N. Y. Ninhydrin (0.2%) in *N*-butanol was a Mann Spraytec Reagent produced by Mann Research Labs, Inc., New York. BBL organic media, Falcon tissue culture glassware and all A. R. grade reagents were purchased from Fisher Scientific Co., Pittsburgh, Pa.

Human skin fibroblasts. Cell lines were initiated from skin biopsies from six normal and six cystinotic children (Table I). Surgical biopsies from normal children were obtained during hernia repair from the inguinal area. Biopsies from cystinotic children were obtained by high-speed punch drill from the lateral upper outer quadrant of a buttock. Small pieces of teased skin were affixed to the surface of 25-cm^2 Falcon flasks and fed with MEM containing 20% fetal bovine serum, 2 mM glutamine, and a mixture of 100 U penicillin and 100 μg streptomycin/ml. Once fibroblasts became evident (in 5–7 days), the cells were refed. By the third feeding (after approximately 2½ wk), antibiotics were omitted from the complete MEM media. Cells were passed in a 1:2 or 1:3 split depending on the growth characteristics of the particular cell line. The general procedure in passing cells from each 32-oz Falcon flask involved washing the culture with 10 ml Puck's solution B followed by trypsinization with 4 ml Puck's solution B containing 0.04% trypsin.

All of the cystinotic patients were of the nephropathic type and exhibited various manifestations of the renal Fanconi syndrome. They all had cystine crystals identified in their corneas and, in all but J. B., (Table I) in rectal mucosa. All patients except A. M. (Table I) were on electrolyte supplements to correct acidosis. A. M. did not require such therapy until age 15 mo. Only J. B. had moderately severe renal failure. M. S. and J. E. had slight elevation of blood urea nitrogen. Normal skin was obtained from healthy children undergoing inguinal hernia repair who were in the same age range as the cystinotic subjects.

Cell lines were checked for mycoplasma (PPLO) by the orcein staining technique of Fogh and Fogh (7) and the agarose method (8). The Hayflick and Moorhead method (9) was employed anaerobically and aerobically. Periodically, cells were monitored for PPLO by electron microscopic examination. The lines were checked also for other microbial contaminants by subculturing a portion of the cell-medium suspension in yeast, mold, and and bacterial broth media together with streaking onto blood agar plates. Only cell lines that were negative by the standard methods of testing for microbial contamination and PPLO were used in experiments.

Cell seeding and growth. Before the start of studies with cells grown on cover slips, a normal and a cystinotic line of comparable passage number from children of approximately the same age were subcultured simultaneously into 32-oz Falcon flasks. These flasks were seeded at such cell densities that both cell lines reached confluency within 7 days. Sterilized 22 × 22-mm cover slips were placed in individual 35 × 10-mm sterile petri dishes and were seeded with about 5×10^4 normal cells or 7×10^4 cystinotic cells. Cells were counted with a Spencer Bright-line Improved Neubauer 1/10 mm deep counting chamber (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). Each petri dish contained 2 ml of MEM with 20% fetal bovine serum and 2 mM glutamine. Incubation was at 37°C

[1] *Abbreviations used in this paper:* DR, distribution ratio; ECF, extracellular fluid; ICF, intracellular fluid; MEM, minimum Eagle's medium; NEM, *N*-ethylmaleiimide; PPLO, mycoplasma; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

under air: CO_2 (95:5). The cells were confluent on the cover slip after 5 days and were refed 24 h before use in an experiment. In transport experiments with 32-oz Falcon flasks, seeding was increased to approximately 5×10^6 cells to assure cell confluency in 5 days. In such experiments, incubation and refeeding were the same as in studies with cells grown on cover slips.

Experimental procedure. The procedure used to determine intracellular fluid space (ICF) was a modification of the method of Foster and Pardee (10), in which comparable cover slips of cells were incubated simultaneously at 37°C for 20 min in 1-ml Dulbecco phosphate-buffered saline (PBS) pH 7.45, containing 0.1% glucose with either 1.0 mM [^{14}C]urea (2 μCi/ml) or 1.0 mM [^{3}H]mannitol (4 μCi/ml). At the end of the incubation, the cover slip was removed from the labeled medium with forceps, rinsed three times with approximately 8 ml/wash ejected from a wash bottle of 37°C 0.9% NaCl, dried on filter paper, and placed in a scintillation vial to which 10 ml of Scintisol-Complete was added. Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (11) applied directly to cells on coverslips that had been rinsed five times with approximately 8 ml/wash of 0.9% NaCl, and air-dried. All calculations of ICF-extracellular fluid (ECF) were based on averages of quadruplicate to sextuplicate determinations, and protein per cover slip was based on an average of 7–10 individual protein determinations. Nanoliters of ICF per microgram protein and nanoliters ECF per microgram protein were calculated for each transport experiment according to the method of Foster and Pardee (10).

Transport studies were conducted in the following manner: Each cover slip confluent with cells was washed once with 1 ml of Dulbecco PBS pH 7.45 containing 0.1% glucose and incubated at 37°C for 30 min in 1 ml fresh Dulbecco PBS + 0.1% glucose. To initiate an experiment, the cover slip was transferred to a preincubated petri dish that contained 1 ml of Dulbecco PBS + 0.1% glucose medium with 0.08 mM L-[^{35}S]cystine (approximately 2.0 μCi/ml). Each cover slip was incubated at 37°C with periodic manual swirling. At the end of the prescribed experimental period, the cover slip was removed with forceps and was rinsed three times with approximately 8 ml/wash of 0.9% NaCl. Between each rinse, excess wash was removed by first touching the edges of the cover slip onto filter paper and then laying the cover slip face-up on filter paper. After the final rinse, the cover slip was placed on a clean filter paper for drying. This entire operation from removal of the cover slip from its petri dish to placement on clean filter paper took approximately 30 s. The dried cover slip was placed in a vial to which 2 ml of Packard Phosphor scintillator, prepared by adding 160 ml of Liquifluor to 3.9 liters of Fisher Scintanalyzed toluene, were added for counting in a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). 10 μl of the incubation media were spotted onto cover slips, dried, and counted simultaneously with confluent cell cover slips with an efficiency of 20%.

The distribution ratio (DR) was calculated as follows:

$$\text{Total cpm } ^{35}\text{S in ECF} = \left(\frac{\text{nl ECF}}{\mu\text{g protein}}\right)\left(\frac{\text{total } \mu\text{g protein}}{\text{on cover slip}}\right)\left(\frac{\text{cpm } ^{35}\text{S}}{\text{nl medium}}\right)$$

$$\text{Total cpm } ^{35}\text{S in ICF} = \text{total cpm } ^{35}\text{S on cover slip} - \text{total cpm } ^{35}\text{S in ECF}$$

$$\frac{\text{cpm } ^{35}\text{S in ICF}}{\text{nl}} = \frac{\text{Total cpm } ^{35}\text{S in ICF/total } \mu\text{g protein}}{\text{nl ICF/}\mu\text{g protein}}$$

$$\text{DR} = \frac{\text{cpm } ^{35}\text{S in ICF/nanoliter}}{\text{cpm } ^{35}\text{S in ECF/nanoliter}}$$

The counts per minute ^{35}S in ECF per nanoliter is based on equilibration of label in ECF with incubation medium and is represented by counts per minute ^{35}S per nanoliter of medium. The DR as calculated is a ratio of radioactivity and is a general indicator of cystine uptake. The nature of the intracellular ^{35}S-containing compounds is shown under Results. All determinations were averages of quadruplicate to quintuplicate samples that varied ±10%.

In efflux studies, the cover slips were incubated for 20 min at 37°C in labeled media, as described above. At the end of the incubation period each cover slip was removed, washed carefully three times with approximately 8 ml/wash 0.9% NaCl, and transferred to 3 ml Dulbecco PBS + 0.1% glucose containing no cystine. 0.1 ml media samples were withdrawn at 3-min intervals and placed directly in a scintillation vial. At the end of 21 min, the coverslip was removed from the unlabeled media, washed three times with 0.9% NaCl, dried on filter paper and placed in a scintillation vial with 2 ml Phosphor for counting in a Packard Scintillation Counter. The percent of radioactivity remaining in the cells was calculated as described by Thier, Segal, Fox, Blair, and Rosenberg (12). Determinations of each study were in triplicate to sextuplicate, depending on the number of cover slips available.

When 32-oz Falcon flasks were substituted for cover slips in efflux experiments, three confluent flasks of a paired cystinotic and normal line were incubated at 37°C for 20 min with 10 ml 0.12 mM L-[^{35}S]cystine, the radioactive media were removed at the end of incubation, and the monolayer of cells was washed three times with 10 ml of 37°C 0.9% NaCl. 6 ml of PBS containing 0.1% glucose were added to each flask and 0.2-ml portions were withdrawn at 3, 6, 9, 12, 15, 18, and 21 min for counting in a Packard Scintillation Counter. At the end of efflux, each cell monolayer was washed twice with 10 ml of 0.9% NaCl, once with 5 ml of Puck's solution B and trypsinized with 5 ml of Puck's solution C. An 0.2-ml portion of the trypsinized cell suspension was counted to determine the ^{35}S remaining within the cells. Calculation of percentage of radioactivity remaining within the cells vs. time was the same as described for cover slip experiments.

In studies of the effects of different concentrations of L-cystine on transport in normal and cystinotic fibroblasts, cells grown on cover slips were incubated for 20 min with different concentrations of L-[^{35}S]cystine as described above for transport studies. 1/V was calculated as the reciprocal of the DR per minute times the millimoles of L-cystine, and apparent K_t and V_{max} were derived from Lineweaver-Burk plots.

Determination of intracellular cyst(e)ine. Intracellular levels of cyst(e)ine in normal and cystinotic cells were determined either by the colorimetric method of States and Segal (13) or with the Beckman 120C Amino Acid Analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The latter method involved homogenization of approximately 20×10^6 cells in 1 ml of 3% sulfosalicyclic acid, followed by freezing and thawing three times and centrifugation at 5,000*g* for 10 min to obtain the cellular extract used for cystine analysis. This analysis was performed with a standard Beckman UR-30 150-cm acidic-neutral column attached to the Analyzer.

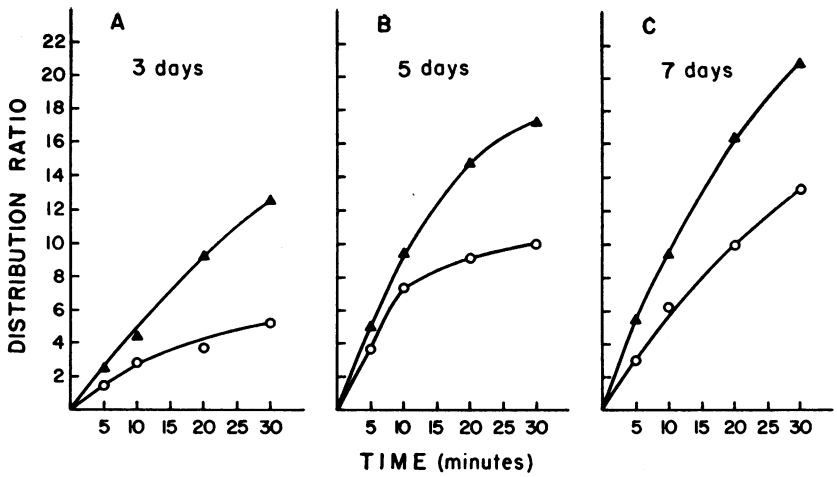

FIGURE 1 Uptake of 0.08 mM L-[^{35}S]cystine by cystinotic T. L. (▲) and normal S. L. (○) fibroblasts in passage 15 grown on cover slips for 3, 5, and 7 days. Values are averages of sextuplicate determinations.

Identification of ^{35}S and determination of specific activity of L-[^{35}S]*cystine.* Attempts to pool sufficient efflux media from cover slip experiments to calculate accurately the specific activity of effluxed L-[^{35}S]cystine proved fruitless because the amount of L-cystine was either below or on the borderline of sensitivity of the Beckman 120C Amino Acid Analyzer. To overcome this difficulty, the following procedures were adopted: Six 32-oz Falcon flasks confluent with cells grown for 5 days were washed with three 10-ml portions of 37°C Dulbecco PBS containing 0.1% glucose. The excess wash was removed by taping each flask several times and the cells were incubated at 37°C for 20 min with 10 ml of Dulbecco PBS + 0.1% glucose containing 0.12 mM L-[^{35}S]cystine. After incubation, the radioactive medium was removed, the monolayer of cells was washed with three 10-ml portions of 0.9% NaCl and drained after each wash, and 3 ml of 37°C Dulbecco PBS + 0.1% glucose were added to each flask. Six flasks of each paired cystinotic and normal line were treated in this manner. Experiments were planned so that pooling of 3- and 21-min efflux media from two 32-oz Falcons into 3 ml of 40 mM NEM contained in 0.01 M phosphate buffer pH 7.5 occurred at 0.5-min intervals with simultaneous 1-min mixing and addition of 0.2 ml 50% trichloroacetic acid (TCA). The NEM-TCA-treated efflux media from each study were pooled and unreacted NEM and TCA were removed by extraction with three 25-ml portions of anhydrous ether. The aqueous phase was evaporated to dryness in vacuo, was resuspended in 1 ml of distilled water and was frozen until analyzed. The monolayer of cells that had been incubated at 37°C for 20 min with 0.12 mM L-[^{35}S]cystine was washed three times with 10 ml 0.9% NaCl. Each flask was drained, and the cells were fragmented and intracellular sulfhydryl groups were reacted with 4 ml of 40 mM NEM in 0.01 M phosphate buffer, pH 7.5, while the monolayer of cells was detached from the surface of the flask by vigorous scraping. The fragmented cell suspensions from each flask were pooled, mixed for 1 min with a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.), and deproteinized with 2 ml of 50% TCA. The rest of the procedure with the cell extract was the same as described above with pooled efflux media. Additional experiments involved resuspension of the pellets in 1 ml of 10% TCA and isolation of the acid-precipitable protein according to the method described by Merchant, Kahn, and Murphy (14) to determine the percent of total label found in the cells that was either exchanged with preexisting -SS- or -SH groups or incorporated into protein.

To separate L-[^{35}S]cystine and its metabolites, a small portion of each sample initially was chromatographed by two methods: First, 20 μl of each sample were spotted onto a cellulose thin-layer plate and chromatographed in Solvent B4 according to the method of States and Segal (15) with standards of cystine, cysteine-NEM and glutathione-NEM spotted and run simultaneously. After the standards were stained with ninhydrin, each plate was cut into 0.5-cm wide sections from 2 cm before the origin to the solvent front. Each section was placed in a vial with 2 ml dilute Phosphor for counting in a Packard scintillation counter. Second, 0.75 ml of each sample was injected onto the neutral-acidic column of the Beckman 120C Amino Acid Analyzer described above. The radioactive effluent from the column was passed through a 2-ml anthracene flow cell (Packard Model 3042 Flow Cell Adapter Assembly) inserted into a Packard Model 2002 ambient temperature Tri-Carb. Based on several amino acid analyzer runs of standard L-[^{35}S]cystine of predetermined activity, the anthracene cell was found to have a counting efficiency of 50% with a background of 50 cpm. Radioactive peaks were recorded on an attached automatic Honeywell Electronik 17 Chart Recorder (Honeywell, Inc., Test Instruments Div., Denver, Col.). When the efflux media were analyzed by the two methods, the percentages of total ^{35}S in cystine were comparable. However, when cell extracts were analyzed, there was excessive tailing of the sulfur amino acids in the thin-layer chromatographic method resulting in poor separation of GSH-NEM from cystine. (The chromatographic separation described by us previously, i.e. States and Segal (15), has interference apparently from high salt and protein concentrations.) Therefore, all of the data on the nature of intracellular and effluxed ^{35}S compounds reported in our results were obtained by column chromatography on the

Beckman 120C Amino Acid Analyzer with ^{35}S assayed by flow counting, as described above.

RESULTS

Standardization of transport measurements. Foster and Pardee (10), whose method we have adapted, pointed out a number of variables which influence the measurement of amino acid uptake by cells on cover slips. These include confluency of cells and fluid spaces. Additional variables in comparing several lines of normal human and cystinotic cells concern the differing rates of division of disparate cell lines as a consequence of changing environmental conditions, inherent growth characteristics of cells, and aging. To insure uniformity of transport measurements, the influence of these factors was studied as well as day-to-day reproducibility.

In initiating cystinotic cell lines from biopsies, we observed that generally these cells divided more slowly than normal fibroblasts grown under the same culture conditions. The time after seeding until growth to confluency and its relation to cystine uptake was therefore determined. Fig. 1 shows the results of transport studies with normal and cystinotic lines in the fifteenth passage 3, 5, and 7 days after cover slips were seeded with equal numbers of cells. Although the figure shows that uptake of L-[^{35}S]cystine was greatest on the 7th day, microscopic examination of the confluent cover slips revealed a substantial number of dead cells and cellular debris, especially in the normal line. On the other hand, when cells were grown for 3 days, the rate of cystine uptake was slowest, neither cell line having reached confluency, with pronounced variability from one cover slip to another. However, on the 5th day, microscopic examination of the cover slips revealed that only the normal cell line had reached confluency. The decision was made, therefore, to seed cover slips with approximately 7×10^4 cystinotic cells and with 5×10^4 normal cells in order to achieve confluency of both types of cells by the 5th day. When confluent with both normal and cystinotic cells, the cover slips contained approximately 100 μg protein. All studies were therefore performed with cells grown on cover slips for 5 days, i.e., when the cover slips were confluent with 90–95% of the cells viable.

The extracellular or mannitol space ranged from 18 to 22% of the total fluid space and did not differ significantly between normal and cystinotic cells. The ICF space, calculated from the difference of urea and mannitol spaces and expressed as nanoliter ICF per microgram cellular protein, varied from one cell line to another (Table I). Cystinotic fibroblasts had about 50% larger ICF space than normal cells at comparable passage. This table also shows that the ICF volume per microgram protein decreases as the cells go into later

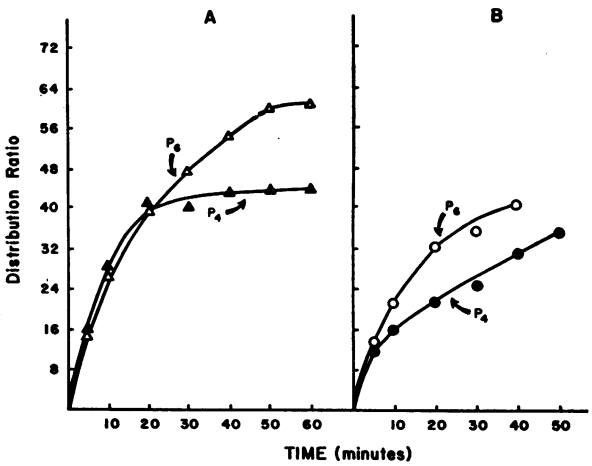

FIGURE 2 Comparison of uptake of 0.08 mM L-[^{35}S]-cystine by cystinotic J. E. (A) and normal F. G. (B) fibroblasts in passage 4 (P_4) (▲, ●) and P_6 (△, ○) grown on cover slips for 5 days. Values are averages of quadruplicate determinations.

passage. Determination of ICF and ECF space was assessed for each cell line at the time of influx and efflux studies.

Fig. 2 shows the uptake of L-[^{35}S]cystine by cystinotic cells (A) and normal cells (B) at the fourth and sixth passages. The rate of uptake for the first 20 min in the cystinotic and the 1st 5 min in the normal line does not differ substantially in passages 4 and 6. The amount of ^{35}S in the cells on longer incubation is more variable, with passage 6 cells accumulating ^{35}S to a greater extent. The uptake of L-[^{35}S]cystine by passage 8 of the cell line of Fig. 2A is shown in Fig 4A. The uptake of ^{35}S appears to be slower and less extensive in passage 8 than in passage 4 and passage 6. The lowest uptake of confluent cells was seen in passage 15 of both a normal and cystinotic line. These findings suggest that in the

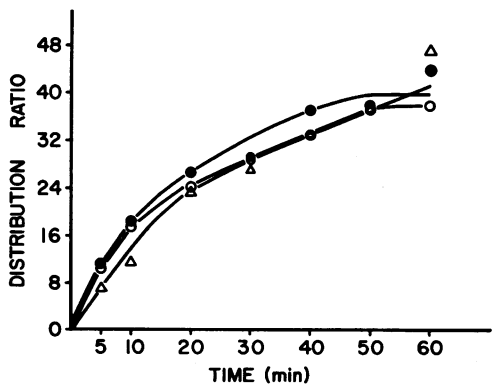

FIGURE 3 Comparison of uptake of 0.08 mM L-[^{35}S]-cystine by a normal cell line in passage 6 seeded onto cover slips on 3 consecutive days and grown for 5 days.

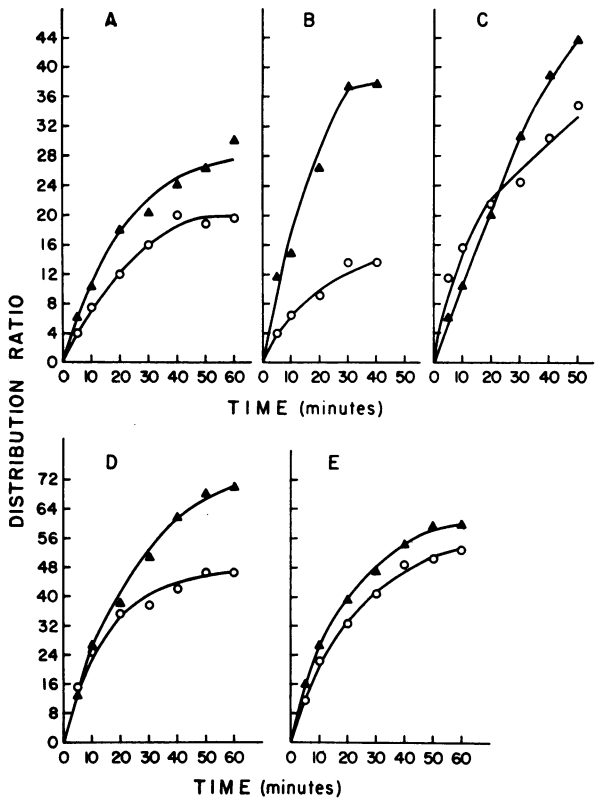

FIGURE 4 Uptake of 0.08 mM L-[^{35}S]cystine by paired cystinotic (▲) and normal (○) cells grown for 5 days on cover slips. The donors and the passage number of the cell lines are: A, cystinotic J. E., normal B. S., P_8; B, cystinotic M. M., normal F. G., P_4; C, cystinotic M. S., normal F. G., P_4; D, cystinotic A. M., normal V. B., P_5; E, cystinotic J. E., normal S. H., P_6. Except for B, each cover slip contained approximately 100 μg protein; cover slips in B contained approximately 55 μg protein. All values are the averages of quadruplicate determinations.

comparison of cell uptake of L-[^{35}S]cystine by normals and cystinotics that cells of the same passage number be employed. They also indicate that late passage cells may differ in their capacity for cystine uptake from those in early passage.

As shown in Fig. 3, there is no significant difference in uptake of 0.08 mM L-[^{35}S]cystine when a cell line is subcultured so that cells in the same passage number, grown under the same environmental conditions, are studied for amino acid uptake on 3 consecutive days. These data confirm the findings of Foster and Pardee (10) and substantiate the advisability of conducting studies with paired cell lines grown under comparable conditions.

Uptake of L-[^{35}S]*cystine.* Once measurements of transport were standardized, uptake studies were undertaken with paired cell lines. Fig. 4 shows the uptake of L-[^{35}S]cystine from media containing 0.08 mM substrate by such paired normal and cystinotic fibroblasts of comparable passage. In all experiments the cystinotic cells achieved a higher distribution ratio than the normal cells. There was considerable variation in the patterns of uptake. In two of the studies (Figs. 4C and 4D) the early uptake (0–20 min) did not differ substantially in the two cell lines. In the other three (Figs. 4A, 4B, and 4E) there appeared to be a difference.

The variability in the extent of uptake of both normal and cystinotic cells may be due to inherent differences in the cell lines, the ages of the cells, and growth conditions at the time of each experiment. The highest distribution ratio was obtained in cells of passages 5 and 6 of Figs. 4D and 4E, lower in cells of passage 8 (Fig. 4A). This may be related to the phenomena shown in Fig. 2. Because of the variable extent of uptake in each study, a plot of the data from all the experiments on a single graph as done for cultured leukocyte uptake (6) would show overlap of the cystinotics and normals, obscuring the difference discerned by adherence to the technique used here.

Concentration dependence of cystine uptake. The velocity of ^{35}S uptake at increasing L-cystine concentrations was measured and the uptake process shown to adhere to saturation kinetics. This is shown in the Lineweaver-Burk plot in Fig. 5. The apparent K_t of two normal cell lines was 0.043 mM L-cystine and the V_{max} 0.18 mmol/liter cell water per min.

The dependence on substrate concentration of two cystinotic cell lines that showed higher than normal initial uptake rate at 0.08 mM (Figs. 1B and 4A) was also determined at the same time as that of the normal cells. In this case the cystinotic cells were paired with a normal cell line different from those where uptake at

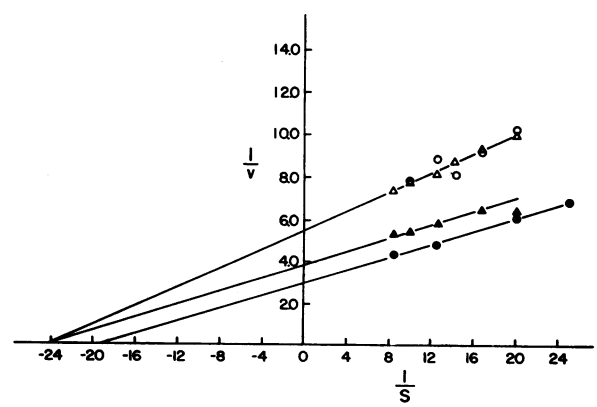

FIGURE 5 Lineweaver-Burk plot showing concentration dependence of cystine uptake by cystinotic J. E. (●) and T. L. (▲) and normals F. G. (○) and V. B. (△). 1/V is the reciprocal of mmoles/liter/minute cystine and 1/S is millimolar cystine^{-1}. Values are averages of sextuplicate determinations.

0.08 mM was compared. At all concentrations used, the cystinotic cells had a higher velocity of uptake with a V_{max} of 0.28 and 0.33 mmol/liter per min. The apparent K_t of the cystinotic cells did not differ from normal cells.

Efflux of accumulated ^{35}S. The possibility that the consistent increase in intracellular L-[^{35}S]cystine seen after 20 min of incubation could be a reflection of differences in efflux of label from cells into the incubation medium has been considered. To determine whether this is the case, cells of both normal and cystinotic lines in pairs were incubated for 20 min with L-[^{35}S]cystine, then transferred to media without the labeled compounds. Assay of the media for radioactivity was measured at 3-min intervals with the results of five experiments shown in Fig. 6. The data are plotted on a semilogarithmic scale as percent of the initial radioactivity in the cells at the start of the efflux vs. time. The patterns of the efflux curves differ somewhat in each experiment but in each there is a major difference between normal and cystinotic cells. In all the curves there appears to be an early rapid efflux phase followed by a slower phase, a finding consistent with there being at least two pools of ^{35}S involved. In the early rapid efflux phase between 0 and 12 min, the efflux of ^{35}S is markedly slower in the cystinotic cells

Nature of intracellular ^{35}S compounds. The distribution ratio used as a measure of L-[^{35}S]cystine uptake represents a radioactivity ratio only. In order to make a more accurate assessment of the transport process as well as to gain insight into the abnormality involved in cystine storage, the identity of labeled intracellular compounds was determined. Table II shows the data obtained after a 20-min incubation. In both normal and cystinotic cells, 95–98% of the total cellular radioactivity was present in the soluble cell extract with 85–95% accounted for in cystine, cysteine, and glutathione. 2–5% of the total radioactivity remained in 2.0–3.5 mg of acid-precipitable cell protein. Differences were observed in intracellular distribution of the label in the two types of cells. In the soluble fraction, cystine accounted for 30% of the ^{35}S in cystinotic cells but only 15% in normal cells. In cystinotic cells, this increase in the amount of label in cystine was reflected by a decrease in the percent of label in cysteine or glutathione, i.e., cysteine decreased in cell line J. E. and glutathione decreased in M. M. in comparison with normal lines.

Table II shows also the distribution of intracellular radioactivity after exposure of the cells to radioactive L-cystine for 20 min followed by removal of the radioactive substrate and incubation of cells in buffered saline. After 3 min of efflux of label from normal cells, the percentage of radioactivity in L-cystine had dropped to 2% with no significant change in the percent of label in cysteine and a small increase in the fraction of total ^{35}S in glutathione. After 21 min in the new media,

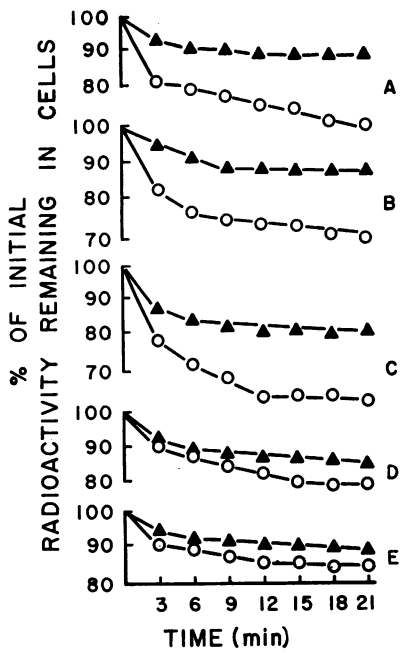

FIGURE 6 Efflux of ^{35}S from paired cystinotic (▲) and normal (○) cells grown on cover slips for 5 days, then incubated with 0.08 mM L-[^{35}S]cystine for 20 min. (see Methods). The cell lines studied are: A, cystinotic J. E., normal T. C., P_8; B, cystinotic M. M., normal F. G., P_4; C, cystinotic J. B., normal S. H., P_4; D, cystinotic A. M., normal V. B., P_6; E, cystinotic T. L., normal B. S., P_6. Values in all but A are averages of sextuplicate determinations. Determinations of A were in triplicate. 100% initial radioactivity = total radioactivity in efflux media corrected to disintegrations per minute (dpm) + radioactivity remaining in cells corrected to dpm. Counting efficiencies were: media 80%, cells 20%.

the amount of ^{35}S in L-cystine was unchanged from the 3 min observation. However, the percentage of ^{35}S in cysteine was reduced by one-half whereas that in glutathione was increased proportionately. The situation in cystinotic cells was quite different from normal cells. The fraction of total ^{35}S in L-cystine decreased from 30% at the end of the uptake phase to 20% after 3 min in the efflux phase. After 21 min in new media, however, the amount of ^{35}S found in L-cystine had returned to 30% of the total label.

In normal cells where the specific activities of intracellular L-[^{35}S]cystine were measured after a 20 min incubation, the average value was 8.71 μCi/μmol 1/2 L-cystine. In the companion cystinotic cell lines, the specific activities of intracellular L-[^{35}S]cystine were 3.21 and 2.70 μCi/μmol 1/2 cystine. The substrate L-[^{35}S]cystine had a specific activity of 9.91 μCi/μmol 1/2 cystine. Within the limits of variability as a result of the methods employed, the isotope dilution seen in the cystinotic cells with a significant intracellular cystine pool correlated well with that expected from calcu-

TABLE II

Identification of Intracellular ^{35}S in Normal and Cystinotic Fibroblasts

Efflux time	Cell line	Patient	NEM adducts				
			L-Cystine	Cysteine	Glutathione	Methionine	Others*
min					*% of total ^{35}S*		
0	Normal	J. W.	15.0	43.0	35.0	0.6	6.4
		S. L.	16.4	40.6	28.5	0.6	10.1
	Cystinotic	M. M.	30.0	38.0	21.0	1.0	8.4
		J. E.	29.1	25.0	31.4	1.0	10.7
3	Normal	J. W.	1.4	41.0	47.0	0.0	8.0
		S. L.	1.6	43.2	40.7	0.0	11.6
	Cystinotic	M. M.	22.0	31.0	39.0	0.0	5.0
		J. E.	18.8	20.0	38.8	0.0	13.6
21	Normal	J. W.	2.2	19.8	68.9	0.0	4.6
		S. L.	1.2	22.3	62.3	0.8	9.2
	Cystinotic	M. M.	29.9	21.4	41.7	0.0	5.0
		J. E.	26.0	10.6	47.9	0.0	10.2

Cells were incubated for 20 min with 0.12 mM L-[^{35}S] cystine contained in Dulbecco PBS +0.1% glucose and transferred to media without label for 3 and 21 min efflux (see Methods). Total ^{35}S recovered in cellular extracts plus acid-precipitable protein after 20-min incubation showed recovery of 96.7%, 98.3%, 98.6%, and 96.4% in the soluble portions from J. W., S. L., M. M., and J. E., respectively. After 3 min efflux, percent of total ^{35}S in the cellular extracts was 95.9, 98.0, 97.7, and 96.2 from J. W., S. L., M. M., and J. E., respectively. After 21 min efflux, values were 93.1%, 96.9%, 97.7%, and 93.8% from J. W., S. L., M. M., and J. E., respectively. The average totals of precipitable protein in each cell line were 2.0, 2.24, 1.90, and 3.45 mg from J. W., S. L., M. M., and J. E., respectively.

* Acidic sulfur amino acids such as taurine, cysteic acid, cysteinesulfinic acid, etc.

lations based on the measured total cell L-cystine and the radioactive material taken up by the cell (4.30 and 4.10 μCi/μmol 1/2 cystine).

Nature of media ^{35}S compounds during efflux. Table III shows the specific activity of L-cystine as well as the pattern of ^{35}S compounds found in the media when cells previously incubated with L-[^{35}S]cystine were placed in substrate-free buffer. The specific activity of the L-[^{35}S]-cystine added to the medium for entry into cells was 9.91 μCi/μmol 1/2 cystine. Cystine in the efflux media

TABLE III

Identity of ^{35}S Compounds Effluxed from Normal and Cystinotic Fibroblasts

Experiment	Cell line	Efflux time	NEM adducts					sp act ½ L-cystine
			L-Cystine	Cysteine	Glutathione	Methionine	Others*	
		min			*% total ^{35}S*			*μCi/μ mol*
Substrate analysis			93.0	0.1	0.1	0.0	4.5	9.91
1.	normal, J. W.	3	74.1	9.7	2.3	2.6	6.0	9.9
	cystinotic, M. M.		77.6	10.2	1.5	2.8	6.0	9.7
	normal	21	76.0	8.4	1.8	2.8	9.3	9.9
	cystinotic		75.0	9.3	1.5	2.7	10.1	10.1
2.	normal, S. L.	3	75.9	12.5	1.6	3.2	5.5	10.4
	cystinotic, J. E.		86.5	2.0	1.0	3.5	5.9	11.9
	normal	21	50.0	23.5	3.2	1.6	20.5	11.3
	cystinotic		67.4	5.2	2.0	2.6	21.0	10.9

* Acidic sulfur amino acids such as taurine, cysteic acid, cysteinesulfinic acid, etc.

Both types of cell lines were incubated simultaneously for 20 min at 37°C with 0.12 mM L-[^{35}S] cystine in Dulbecco PBS + 0.1% glucose. After removal of the radioactive substrate the cells were washed and reincubated for 3 and 21 min in Dulbecco PBS +0.1% glucose without label. The efflux media were treated and pooled as described under Methods. The data in this table are the counterparts of Table II data.

TABLE IV

Changes in Intra- and Extracellular L-[^{35}S]Cystine during Efflux

Cell line	Before efflux		After 3 min of efflux				After 21 min of efflux			
	Cells		Cells		Media		Cells		Media	
	Total	Cystine*‡	Total	Cystine	Total	Cystine	Total	Cystine	Total	Cystine
					nmol × 10^{-2} L-cystine					
Normal										
T. C.	71.68	10.75	58.93	0.88	12.75	9.57	50.95	0.76	20.73	13.06
F. G.	45.00	6.76	36.17	0.54	8.87	6.65	30.91	0.46	14.14	8.91
S. H.	68.68	10.30	53.87	0.81	14.80	11.10	43.98	0.66	24.70	15.56
V. B.	54.40	8.16	49.20	0.74	5.21	3.90	42.90	0.64	11.50	7.24
B. S.	37.30	5.59	33.85	0.51	3.46	2.59	31.33	0.63	5.97	3.76
Mean ±SEM	55.41±6.63	8.31±0.99	46.4±4.91	0.70±0.07	9.02±2.15	6.76±1.62	40.01±3.89	0.63±0.05	15.41±3.32	9.71±2.1
Cystinotic										
J. E.	60.21	17.46	55.90	10.51	4.31	3.73	52.22	13.58	7.99	5.35
A. M.	100.97	30.29	92.48	20.34	8.48	6.59	84.29	23.60	16.67	11.87
M. M.	57.08	17.12	53.35	11.74	3.73	2.90	49.50	14.80	7.58	5.68
T. L.	58.22	17.47	54.77	12.05	3.46	2.68	51.58	14.44	6.64	4.73
Mean ±SEM	69.12±10.64	20.6±3.2	64.13±9.47	13.66±2.25	5.00±1.18	3.98±0.90	59.4±8.32	16.61±2.35	9.72±2.33	6.91±1.7

* Calculated from adjustment to 100 μg protein/cover slip with 2 μCi ^{35}S/0.08 μmol/ml L-cystine or 25 nCi ^{35}S/nmol L-cystine substrate.

‡ Calculations are based on average percent of total label recovered in cystine (Tables II and III) except for "after 3 min of efflux" for A. M. and T. L. when percentages from M. M. are applied: before efflux, normal 15%, cystinotic 30%; 3 min cells, normal 1.5%, cystinotic (A. M. and T. L.) 22%; efflux, normal 75%, cystinoti (A. M. and T. L.) 74.1%; 21 min cells, normal 1.5%, cystinotic (A. M. and T. L.) 28%; efflux, normal 63%, cystinotic (A. M. and T. L.) 71.2%. For cystinotic J. E. and M. M. calculations are based on data presented in Tables II and III.

was found to range in sp act from 9.70 to 11.25 with no difference in values between normal and cystinotic cells (Table III). Indeed, the specific activity of media L-cystine was similar to the substrate L-[^{35}S]cystine. The possibility that this similarity is due to entrapped extracellular L-[^{35}S]cystine can be discounted since, based on our calculations (see Methods), less than 1% of the total L-[^{35}S]cystine taken up by the cells represented the counts in the ECF space after 20 min incubation.

The ^{35}S compounds identified in the efflux media were predominately cystine, with lesser amounts of cysteine and glutathione. Calculation of percentages of total ^{35}S appearing in each compound is shown in Table III. The fact that normal cells show a depletion of intracellular L-[^{35}S]cystine after short-term incubation in substrate-free medium with concomitant presence of labeled L-cystine in the medium confirm that L-cystine is the major compound exiting from the cells, especially at the end of 3 min of efflux. After 21 min efflux, however, a portion of the cysteine effluxed from the cells could be oxidized to cystine.

Correlation of changes in localization of intra and extracellular ^{35}S during efflux. Table IV tabulates total label and L-[^{35}S]cystine recovered within the cells before efflux, and remaining within the cells and in the corresponding media after the 3- and 21-min efflux periods. The calculations are based on the data of Tables II and III, obtained in experiments with 32-oz Falcon flasks, exclusive of small percentages of label in the acid-precipitable cellular protein, as applied to the efflux patterns from cover slip experiments shown in Fig. 6. All values are expressed as nanomoles × 10^{-2} L-cystine, derived from the initial specific activity of L-cystine incubated with cover slips for 20 min. At the end of 3 min of efflux, the period of rapid loss of label from the cells, normal cells have an average loss of 7.61 nmol × 10^{-2} L-cystine compared with 6.77 nmol × 10^{-2} L-cystine found in the efflux media, or a recovery of 89%. For the same efflux period, cystinotic cells have an average depletion of 6.93 nmol × 10^{-2} intracellular L-cystine with 4.00 nmol × 10^{-2} L-cystine accounted for in the efflux media, a recovery of 58%. By the end of a 21-min efflux, the amounts of L-cystine recovered within the efflux media are generally in excess of the calculated amounts of L-cystine disappearing from the cells, i.e. 9.71 and 6.92 nmol × 10^{-2} L-cystine compared with calculated intracellular disappearance of 7.68 and 3.98 nmol × 10^{-2} L-cystine for normal and cystinotic cells, respectively.

Since substantial amounts of ^{35}S were found in metabolites of L-cystine, as set forth in Tables II and III, the total percentages of label (Fig. 6) inside the cells after the 20-min uptake period and inside and outside the cells after the efflux periods were analyzed to determine the distribution of substrate L-cystine in cysteine, GSH and other free sulfur compounds. The results are tabulated in Table V, which also includes the total change in label

TABLE V

Fate of Intracellular L-[^{35}S]

Cell line	Before efflux				After 3 min of efflux									
	Cells*				Cells*			Media*			Net change			
	Total	CSH	GSH	Others	CSH	GSH	Others	CSH	GSH	Others	CSSC‡	CSH	GSH	Others
	nmol × 10^{-2} L-cystine													
Normal														
T. C.	71.68	29.96	22.79	6.38	24.81	25.87	5.77	1.42	0.25	1.11	−0.30	−3.74	3.33	0.50
F. G.	45.00	18.83	14.32	4.01	15.23	15.88	3.55	0.99	0.18	0.77	0.44	−2.62	1.73	0.31
S. H.	68.68	28.71	21.84	6.11	22.68	23.65	5.28	1.64	0.30	1.29	1.61	−4.38	2.11	0.45
V. B.	54.40	22.74	17.30	4.84	20.71	21.60	4.82	0.58	0.10	0.45	−3.52	−1.45	4.40	0.43
B. S.	37.30	15.59	11.86	3.32	14.25	14.86	3.32	0.38	0.07	0.30	−2.49	−0.96	3.07	0.00
Mean	55.41	23.17	17.62	4.93	19.54	20.37	4.55	1.00	0.18	0.78				
±SEM	±6.63	±2.77	±2.11	±0.59	±2.07	±2.16	±0.48	±0.24	±0.04	±0.19				
Cystinotic														
J. E.§	60.21	15.05	18.91	7.05	11.18	21.69	7.60	0.09	0.04	0.41	−3.22	−3.79	2.83	0.96
A. M.	100.97	31.80	26.45	10.70	28.67	36.06	4.60	0.87	0.13	0.77	−3.36	−2.26	9.74	−5.35
M. M.§	57.08	21.69	11.99	5.37	16.54	20.81	2.67	0.38	0.06	0.33	−2.49	−4.77	8.87	−2.37
T. L.	58.22	18.34	15.25	6.17	16.98	21.36	2.74	0.35	0.05	0.31	−2.74	−1.71	6.16	−3.13
Mean	69.12	21.72	18.15	7.32	18.34	24.98	4.40	0.42	0.07	0.46				
±SEM	±10.64	±3.62	±3.11	±1.18	±3.69	±3.70	±1.16	±0.16	±0.02	±0.11				

* See Table IV.

‡ Calculated from data in Table IV.

§ J. E. and M. M. calculations are based on data presented in Tables II and III.

‖ Balance = total increase in net change of compounds/total decrease in net change of compounds × 100. Calculations are based on percent of total label recovered as described in Table IV. Before efflux—GSH—normal 31.8%, cystinotic 26.2%; cysteine (CSH)—normal 41.8%, cystinotic 31.5%; others (see Table II)—normal 8.9%, cystinotic 10.6%. 3 min—cells—CSH—normal 42.1%, cystinotic 31%; GSH—normal 43.9%, cystinotic 39%; others—normal 9.8%, cystinotic 5%. 3 min—(efflux media)—CSH—normal 11.1%, cystinotic 10.2%; GSH—normal 2.0%, cystinotic 1.5%; others—normal 8.7%, cystinotic 8.8%. 21 min—cells—CSH—normal 21.1%, cystinotic 16%; GSH—normal 65.6%, cystinotic 44.8%; others—normal 7.3%, cystinotic 7.6%. 21 min—(efflux media)—CSH—normal 16%, cystinotic 3.6%; GSH—normal 2.5%, cystinotic 1.5%; others—normal 17.1%, cystinotic 18.2%.

as determined by intra- and extracellular recoveries after 3 and 21 min of efflux, and the percentages of ^{35}S accounted for at the end of the two efflux periods. The data show that L-[^{35}S]cystine is continuously incorporated, via cysteine, into glutathione in normal cells during the entire efflux period. Cystinotic cells, on the other hand, incorporate ^{35}S from L-cystine twice as rapidly into glutathione as normal cells during the first 3 min of efflux. However, by the end of 21 min of efflux, additional L-[^{35}S]cystine appears to have been generated within the cell (Table IV) and the increase in [^{35}S]-glutathione from the value found after the initial 20 min L-[^{35}S]cystine preloading period is comparable with that seen in normal cells (Table V). The recovery of label in normal cells approximates 95% for both efflux periods. In cystinotic cells, the loss of 8.5% of the label in the cell extract of J. E. after 3 min of efflux in the NaOH wash from the amino acid column may account for the low recovery of 54% of the total label. It is possible also that the rate of attaining a new steady state in the cystinotic lines may differ from one cell line to another, as evidenced by 81 and 89% recoveries for 3-min of efflux and 89 and 88% for 21-min of efflux in T. L. and A. M., respectively.

Table V also shows the change in labeled cysteine during the 21-min efflux period. There appeared to be a loss of ^{35}S from the cysteine pool, which was not different in the two types of cells (2.63 and 3.14 nmol × 10^{-2} for normal and cystinotic cells respectively after 3 min efflux; 12.30 and 11.76 nmol × 10^{-2}, respectively, after 21 min.

A comparison of the calculated disappearance of cystine from the cells (cell cystine before efflux and cell cystine after 21 min) at the end of efflux (Table IV) with the media cystine after 21 min efflux (Net change cystine, Table V) shows an increase in cystine in the system for most of the normal and cystinotic cell lines. Such a net positive change was not observed after 3 min of efflux, suggesting that the net cystine increase after 21 min could be due to cysteine oxidation in the oxidative environment of the efflux media.

DISCUSSION

The availability of fibroblasts with the phenotype of increased cystine content from cystinotic patients has made these cells an attractive model for studying the etiology of cystine storage disease. Studies with these cells have thus far been meager. Hummeler, Zajac, Genel, Holtzapple, and Segal (16) have shown that cystinotic fibroblasts have many inclusion bodies of amorphous material surrounded by a membrane, a finding that corresponds to the amorphous material and crystals believed

	After 21 min of efflux										
	Cells*			Media*			Net change				
Balance‖	CSH	GSH	Others	CSH	GSH	Others	CSSC	CSH	GSH	Others	Balance‖
%	*nmol* × 10^{-2} *L-cystine*										%
95	10.75	33.43	3.72	3.32	0.52	3.54	3.07	−15.90	11.15	0.88	95
95	6.52	20.27	2.26	2.26	0.35	2.42	2.62	−10.04	6.30	0.67	95
95	9.28	28.85	3.21	3.95	0.62	4.22	5.92	−15.48	7.63	1.32	96
97	9.05	28.15	3.13	1.84	0.29	1.97	−0.27	−11.85	11.13	0.26	94
89	6.61	20.55	2.29	0.60	0.09	0.64	−1.21	−8.38	8.78	−0.39	88
	8.44	26.25	2.92	2.39	0.37	2.56					
	±0.82	±2.55	±0.28	±0.58	±0.09	±0.62					
54	5.54	25.02	5.33	0.42	0.16	1.89	1.47	−9.10	6.27	0.77	94
89	13.49	37.76	6.41	0.60	0.25	3.03	5.18	−17.72	11.56	−1.26	88
92	10.59	20.64	2.47	0.71	0.11	0.97	3.36	−10.39	8.77	−1.92	99
81	8.25	23.11	3.92	0.24	0.10	1.21	1.70	−9.85	7.95	−1.04	89
	9.47	26.63	4.53	0.49	0.16	1.78					
	±1.69	±3.82	±0.86	±0.10	±0.03	±0.46					

to be contained in structures with the characteristics of lysosomes (17). Schulman and Bradley have shown that the swelling of lysosomes when cells are incubated with disulfides is related to cystine (2). Recently, there has been a report that the cysteine and GSH content of cystinotic fibroblasts is within the normal range (3).

In 1965, because of reports in the literature of the inability to show a defect in cystine metabolism, Segal (18) suggested that the disorder may involve membrane transport of cystine, especially a defect in efflux of the sulfur amino acid from cells. The present studies were undertaken to investigate this hypothesis.

To investigate transport parameters in cultured fibroblasts, a suitable technique was required. There appears to be a choice of two techniques. The first is the use of fibroblast suspensions prepared by treatment of monolayer cultures with 0.25% trypsin (19). The second is the use of monolayers on glass cover slips (20). We chose the second alternative because of the possible alteration of cell membranes by trypsin. Shen and Ginsburg (21) had shown that under normally used conditions of trypsinization of monolayer cultures, from 31 to 47% of galactose, mannose, fucose, glucosamine, galactosamine, and *N*-acetyl-neuraminic acid was released from HeLa cells. This carbohydrate material is probably derived from cell surface glycoproteins.

Foster and Pardee (10) extensively evaluated the characteristics of the cover slip technique and reported that in comparing two cell lines there was variability of rates of accumulation in different series of experiments, but that with a series of experiments with cells seeded on the same day in the same batch of growth medium, results were reproducible. We are aware that differences in growth rates might be due to differences in the sites of skin biopsie, i.e., buttock area from cystinotic patients and inguinal area from normal controls, but we have minimized variation in cell comparisons by conducting experiments with a normal and cystinotic line in tandem grown in the same media, seeded at the same time, and in the same passage. Also, cover slips were more heavily seeded with cystinotic than normal cells, so that asynchronous cell populations would reach confluency and be predominantly in the early resting phase of the cell cycle in 5 days to circumvent differences in growth rates between the two types of cells. All of our control cultures were grown from children of approximately the same ages as the cystinotic patients.

The results have been expressed on the basis of labeled material per milliliter of ICF. From this we have calculated the DR, an index commonly used in transport studies to measure uptake of a substrate into cells. Uptake has been expressed on the basis of amount of sub-

strate per microgram of protein (10). The cystinotic cells have up to twice as much ICF per microgram protein as normal cells. In this case with the same amount of protein on cover slips from the two types of cells, calculation of uptake on the basis of protein would alter the relationship of the uptake curves of cystinotic cells to normal cells in Figs. 1, 2, and 4, exaggerating the differences by a factor up to two.

Groth and Rosenberg (22) have used trypsinized cells in suspension to study uptake of cystine by normal fibroblasts and fibroblasts from patients with cystinuria, the disease where urinary cystine is increased. It is difficult to compare their uptake curves with ours since the age and passage of their cells were not given. The rate of uptake of the cells in suspension seemed about half that of our cells in passage 4 or 6 and more like that of our older cells. The K_m for cystine uptake in trypsinized cells was 0.71 mM compared to the more physiological level of 0.043 mM in cells in monolayer.

In the present studies the original intent was to study membrane parameters of cystine transport in cultured fibroblasts, much as we have studied them in normal kidney cortex slice (23) and intestinal segments (24). Since solutes are normally in a dynamic equilibrium across cell membranes (25, 26), intracellular levels depend on the velocity of entry as well as efflux. Delineation of the influx and efflux components in this type of experiment depends on the transported substrate being in a free state in the cellular compartment and the rate of metabolism of the transported substrate being slow relative to the entry and exit rates. These considerations prompted the experiments reported here, which were conducted with the realization that the cystinotic cells we were dealing with differed from the normal, in that they possessed a sequestered cystine pool.

The standardized procedure employing cells sheeted on cover slips to evaluate cystine uptake revealed a greater accumulation of ^{35}S compounds by cystinotic than by normal cells. The assessment of the dependence of uptake on external cystine concentration by Michaelis-Menten analysis indicated that entry was indeed concentration-dependent, with cystinotic cells having the same transport K_t but a higher maximum velocity than normal cells. Since the presumed binding site is considered membrane-associated, it may be concluded that the K_t reflects a valid similarity between the cells. On the other hand, the difference in maximum velocity may not specifically reflect a primary increase in efficiency of the transport system in cystinotic cells but may be the result of metabolic events within the cell resulting in eventual entrapment of the entering cystine.

Our analysis of the intracellular ^{35}S compounds after a 20-min incubation indicates that in both cell types the radioactivity is mainly in cystine, cysteine, and glutathione, with a small fraction, less than 5%, remaining in the acid-insoluble cellular protein fraction. The normal fibroblast differs from other cells, such as those in brain, muscle, intestine, and kidney, where under similar conditions in vitro no intracellular cystine is present (27). The majority of the ^{35}S is in cysteine and glutathione with no consistent difference between cystinotic and normal cells (Table V). Thus, the cystine entering the cell is extensively metabolized by reduction to cysteine, followed by conversion of the latter compound to glutathione. The data reported here are consistent with the previous publication of normal glutathione and cysteine levels in cystinotic fibroblasts (3). Indeed, we[2] along with Tietze, Bradley, and Schulman (28) have found cystinotic cells to have glutathione-cystine transhydrogenase and glutathione reductase activities.

The principle difference between cystinotic and normal cells after 20 min incubation in L-[^{35}S]cystine is that twice as much label is present as L-[^{35}S]cystine in the diseased cells. In fact, it appears that the increase in ^{35}S uptake observed in cystinotic cells may be accounted for by the increased intracellular L-[^{35}S]cystine.

Efflux experiments also were undertaken in the hope of gaining insight into the mechanism whereby cystine is sequestered in cystinosis. The design of these experiments with labeling of the intracellular pools and transfer to medium without cystine is one of pulse-labeling. The studies shown in Fig. 6 indicate that the percent of the total intracellular ^{35}S effluxing from cystinotic cells was less than in normals, especially within the first several minutes. Examination of the intracellular ^{35}S pools after 3 min efflux revealed that in normal cells 90% of the labeled cystine present after 20 min incubation with L-[^{35}S]cystine had disappeared and in three of five cell lines all of this was found in the efflux media. In the cystinotic cells, the labeled cystine pool, representing 30% of the total intracellular ^{35}S after 20 min preloading, decreased by 33% but only about half of this was found in the efflux media. However, that fraction of L-[^{35}S]cystine lost from the cystinotic cell pool and not effluxing could be accounted for by an increase in [^{35}S]glutathione. This increase in incorporation of label into glutathione in cystinotic cells was twice the amount appearing in glutathione in normal cells. After 21 min of efflux, the cystinotic cells had an increase in label in the cystine pool despite the fact that L-[^{35}S]-cystine continued to appear in the medium. The fact that the specific activity of the L-[^{35}S]cystine that appeared in the efflux media was the same as that taken up indicates that the cystine leaving the cell had not equilibrated or exchanged with the lysosomal cystine pool.

With the uptake and efflux data and analysis of intra- and extracellular ^{35}S compounds, there are several pos-

[2] Unpublished observation.

sible explanations for increased intracellular L-[^{35}S]-cystine in cystinotic cells: First, the L-[^{35}S]cystine entering the cell is reduced to [^{35}S]cysteine, which undergoes rapid oxidation to cystine, the latter being stored in the existing lysosomal cystine pool. The enzymatic oxidation of cysteine to cystine has been reported (29), and impaired control of such an enzyme could result in faster oxidation of cysteine to cystine in cystinotic than in normal cells. Second, the efflux of cystine is slowed by alteration of the membrane transport process. Third, the faster incorporation of L-[^{35}S]-cystine into glutathione before establishment of the new steady state at 21 min efflux (Tables IV and V) suggests that in the cystinotic cell the rapid accumulation of GSH in the first 3 min of efflux could lead to accelerated turnover of glutathione with reaccumulation of cystine. Of the possibilities enumerated, the first appears plausible but requires proof from further studies. In regard to the second, the data reveal little difference between normals and cystinotics in actual amounts of cystine leaving the cell; however, the percent of the pool effluxing is markedly diminished from cystinotic cells. This could indicate a defect in efflux if all of the cystine pool were available for efflux. This we cannot determine from our data, since we have no way of knowing how much of the labeled cell cystine pool in the cystinotic is sequestered or associated with the endogenous pool. As to the third possibility, the faster incorporation of label into glutathione during the first 3-min efflux period could explain the decrease in total label effluxed by the cystinotic cells. While the recycling of cysteine from glutathione appears attractive, it will be essential to determine the fate of cysteine derived from glutathione turnover. The importance of glutathione in transport and metabolism has been stressed recently by Meister (30).

Our data suggest that to arrive at a more complete assessment of the etiology of cystine storage disease, further studies should include fractionation of cells after preloading with L-[^{35}S]cystine to determine localization of label; second, investigation of cysteine-glutathione interrelationships; and third, the use for [^{35}S]-cystine metabolic and transport studies of cells that possess the defect of cystinosis but do not possess a large cystine pool. They could be cystinotic cells treated to remove the pool or cells heterozygous for cystinosis that have only a slight increase in cell cystine content.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Robert Reynolds for cystine assays with a Beckman Model 120C amino acid analyzer.

This study was supported by Grant AM 13900 from The National Institutes of Health.

REFERENCES

1. Schneider, J. A., F. M. Rosenbloom, K. H. Bradley, and J. E. Seegmiller. 1967. Increased free-cystine content of fibroblasts cultured from patients with cystinosis. *Biochem. Biophys. Res. Commun.* **29**: 527.
2. Schulman, J. D., and K. H. Bradley. 1970. The metabolism of amino acids, peptides, and disulfides in lysosomes of fibroblasts cultured from normal individuals and those with cystinosis. *J. Exp. Med.* **132**: 1090.
3. Schulman, J. D., J. A. Schneider, K. H. Bradley, and J. E. Seegmiller. 1972. Cystine, cysteine, and glutathione metabolism in normal and cystinotic fibroblasts *in vitro*, and in cultured normal amniotic fluid cells. *Clin. Chim. Acta.* **37**: 53.
4. Schneider, J. A., K. H. Bradley, and J. E. Seegmiller. 1968. Transport and intracellular fate of cysteine-^{35}S in leukocytes from normal subjects and patients with cystinosis. *Pediatr. Res.* **2**: 441.
5. Rosenberg, L. E., and S. J. Downing. 1965. Transport of neutral and dibasic amino acids by human leukocytes: absence of defect cystinuria. *J. Clin. Invest.* **44**: 1382.
6. Schulman, J. D., K. H. Bradley, I. K. Berezesky, P. M. Grimley, W. E. Dodson, and M. S. Al-Aish. 1971. Biochemical, morphologic, and cytogenetic studies of leukocytes growing in continuous culture from normal individuals and from patients with cystinosis. *Pediatr. Res.* **5**: 501.
7. Fogh, J., and H. Fogh. 1964. A method for direct demonstration of pleuropneumonia-like organisms in cultured cells. *Proc. Soc. Exp. Biol. Med.* **117**: 899.
8. Sedwick, W., and T. J. Wiktor. 1967. Reproducible plaquing system for rabies, lymphocytic choriomeningitis and other BHK-21/135 agarose suspensions. *J. Virol.* **1**: 1224.
9. Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**: 585.
10. Foster, D. O., and A. B. Pardee. 1969. Transport of amino acids by confluent and non-confluent 3T3 and polyoma virus-transformed 3T3 cells growing on glass cover slips. *J. Biol. Chem.* **244**: 2675.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
12. Thier, S. O., S. Segal, M. Fox, A. Blair, and L. E. Rosenberg. 1965. Cystinuria: defective intestinal transport of dibasic amino acids and cystine. *J. Clin Invest.* **44**: 442.
13. States, B., and S. Segal. 1973. Quantitation of cys-t(e)ine in human fibroblasts and separation of cysteine-sulfinic acid, cysteic acid and taurine. *Clin. Chim. Acta.* **43**: 49.
14. Merchant, D. J., R. H. Kahn, and W. H. Murphy. 1960. Handbook of Cell and Organ Culture. Burgess Publishing Company, Minneapolis, Minn. 162.
15. States, B., and S. Segal. 1969. Thin-layer chromatographic separation of cysteine and the *N*-ethyl-maleimide adducts of cysteine and glutathione. *Anal. Biochem.* **27**: 323.
16. Hummeler, K., B. A. Zajac, M. Genel, P. G. Holtzapple, and S. Segal. 1970. Human cystinosis: intracellular deposition of cystine. *Science (Wash., D. C.).* **168**: 859.
17. Patrick, A. D., and B. D. Lake. 1968. Cystinosis:

electron microscopic evidence of lysosomal storage of cystine in lymph node. *J. Clin. Pathol. (Lond.).* **21:** 571.
18. Segal, S. 1965. Disorders of amino acid transport. *Ann. Intern. Med.* **62:** 847.
19. Mahoney, M. J., and L. E. Rosenberg. 1970. Uptake of α-aminoisobutyric acid by cultured human fibroblasts. *Biochim. Biophys. Acta.* **219:** 500.
20. Platter, H., and G. M. Martin. 1966. Tryptophan transport in cultures of human fibroblasts. *Proc. Soc. Exp. Biol. Med.* **123:** 140.
21. Shen, L., and V. Ginsburg. 1968. Release of sugars from HeLa cells by trypsin. *In* Biological Properties of the Mammalian Surface Membrane. L. Manson and A. Parsons, editors. The Wistar Institute Press, Philadelphia, Pa. 67–71.
22. Groth, U., and L. E. Rosenberg. 1972. Transport of dibasic amino acids, cystine, and tryptophan by cultured human fibroblasts: absence of a defect in cystinuria and Hartnup disease. *J. Clin. Invest.* **51:** 2130.
23. Segal, S., and J. C. Crawhall. 1968. The characteristics of cysteine and cystine transport in the rat kidney. *Proc. Natl. Acad. Sci. U. S. A.* **59:** 231.
24. States, B., and S. Segal. 1968. Developmental aspects of cystine transport in rat intestine. *Biochim. Biophys. Acta.* **163:** 154.
25. Rosenberg, L. E., M. Berman, and S. Segal. 1963. Studies of the kinetics of amino acid transport, incorporation into protein and oxidation in kidney cortex slices. *Biochim. Biophys. Acta.* **71:** 664.
26. McNamara, P., C. Rea, and S. Segal. 1971. Sugar transport: Effect of temperature on concentrative uptake of α-methyl-glucoside by kidney cortex slices. *Science (Wash., D. C.).* **172:** 1033.
27. Crawhall, J. C., and S. Segal. 1967. Intracellular ratio of cystine and cysteine in various tissues. *Biochem. J.* **105:** 891.
28. Tietze, F., K. H. Bradley, and J. D. Schulman. 1972. Enzymic reduction of cystine by subcellular fractions of cultured and peripheral leukocytes from normal and cystinotic individuals. *Pediatr. Res.* **6:** 649.
29. Schneider, J. A., and J. E. Seegmiller. 1972. Cystinosis and the Fanconi syndrome. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 1581.
30. Meister, A. 1973. On the enzymology of amino acid transport. *Science (Wash., D. C.).* **180:** 33.