

## Decrease of Intracellular Cystine Content in Cystinotic Fibroblasts by Inhibitors of $\gamma$ -Glutamyl Transpeptidase\*

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Jean DeBrohun Butler† and Stephen P. Spielberg§

From the Section on Human Biochemical and Developmental Genetics, National Institutes of Child Health and Human Development, Bethesda, Maryland 20205

Cystine content of skin fibroblasts derived from patients with cystinosis was decreased by inhibitors of  $\gamma$ -glutamyl transpeptidase, the initial enzyme in glutathione catabolism. The addition of maleate or the  $\gamma$ -glutamyl hydrazone of  $\alpha$ -ketobutyric acid to culture medium (1–20 mM) resulted in dose-dependent decreases of up to 55% on intracellular cystine content of cystinotic cells in 24 h. L-Serine in sodium borate buffer (40 mM each) produced similar results and further decreased cystine levels to 14% of cystinotic control values after 10 days incubation. Analysis of intracellular amino acids showed that, in general, other amino acids remained unchanged following serine-borate treatment. These results suggest that cystine storage in cystinotic tissues may be related to metabolism of glutathione.

Cystinosis is a human genetic disease associated with cystine storage in a number of tissues and with clinically progressive renal failure (1). Cells isolated from patients may contain up to 100-fold more cystine than normal cells. The source of the cystine, which is localized to lysosomes, is still uncertain (2, 3). Thoene *et al.* (4) have proposed that the cystine may come largely from protein digestion in lysosomes. However, a large amount of cellular cysteine (up to 10 mM) is present in cells in the tripeptide glutathione ( $\gamma$ -glutamyl cysteinylglycine) (5, 6), the breakdown of which could serve as a source of the excess cystine. Glutathione is catabolized in the  $\gamma$ -glutamyl cycle by  $\gamma$ -glutamyl transpeptidase and a nonspecific dipeptidase, releasing cysteine (7, 8). We therefore postulated that inhibition of  $\gamma$ -glutamyl transpeptidase, the first enzyme in glutathione breakdown, might decrease the cystine content of cystinotic fibroblasts in culture. This possibility was tested by introducing a variety of inhibitors of  $\gamma$ -glutamyl transpeptidase into the culture medium. All produced a dose-

related lowering of the cystine content of cystinotic cells.

### MATERIALS AND METHODS

Skin fibroblasts were obtained from cystinotic patients with written informed consent. Normal skin fibroblasts came from the American Type Culture Collection, Rockville, MD and from the Human Genetic Mutant Cell Repository, Camden, NJ. L- $\gamma$ -Glutamyl-*p*-nitroanilide, maleic acid (disodium salt), L-serine, sodium borate, sulfosalicylic acid, *N*-ethylmaleimide, 5,5'-dithiobis(2-nitrobenzoic acid), L- $\gamma$ -glutamyl hydrazide, and  $\alpha$ -ketobutyric acid were purchased from Sigma.  $\gamma$ -Glutamyl hydrazone of  $\alpha$ -ketobutyric acid was produced immediately before use as outlined by Tate and Meister (9). Glutathione reductase and NADPH were obtained from Calbiochem. L-[<sup>14</sup>C]Cystine (295 mCi/mmol) was obtained from New England Nuclear and L-[<sup>35</sup>S]cystine (4 Ci/mmol) from Schwarz/Mann. Cystine-binding protein was a generous gift from Dr. J. A. Schneider, University of California at San Diego, La Jolla, CA. Phosphate buffered saline (0.1 M sodium phosphate buffer, pH 7.4, in 0.15 M NaCl), nonessential amino acids, and Eagle's Minimum Essential Medium were supplied by the National Institutes of Health Media Preparation Department. Heat-inactivated fetal bovine serum was purchased from Grand Island Biological Company, Grand Island, MI.

Cells were maintained by standard methods in Eagle's Minimum Essential Medium supplemented with nonessential amino acids and 10% fetal bovine serum. Cells were trypsinized with 0.25% trypsin in phosphate buffered saline, pH 7.4, plated onto 60-mm dishes containing 5 ml of medium and grown to confluency. For inhibition studies, fresh media containing varying concentrations of inhibitors was added. For those studies using serine-borate as inhibitor, a stock solution of serine in borate buffer (400 mM each) was prepared and appropriate aliquots were added to the medium to produce final concentrations of 1–40 mM. Control medium contained equivalent volumes of phosphate buffered saline. Cells were harvested by trypsinization and the washed drained cell pellets were sonicated in 0.3 ml of *N*-ethylmaleimide (3.2 mg in 5 ml of 50 mM potassium phosphate buffer, pH 7.4). Proteins were precipitated by addition of 0.1 ml of 12% sulfosalicylic acid. After centrifugation for 10 min at 2000 rpm, the supernatants were subjected to cystine analysis by the cystine-binding protein assay of Oshima *et al.* (10). The residual cell pellets were dissolved in 1 ml of 0.5 N NaOH and protein was determined by the method of Lowry *et al.* (11).

As an alternative measurement of intracellular cystine, cells were labeled with L-[<sup>35</sup>S]cystine (10  $\mu$ Ci/ml of medium), harvested, sonicated in the presence of *N*-ethylmaleimide, precipitated with sulfosalicylic acid, and assayed for protein as stated above. The cell supernatant containing *N*-ethylmaleimide adducts of free thiol groups along with cystine and oxidized glutathione was subjected to high voltage electrophoresis (4 kV, 2 h) in 7.8% formic acid using a Gilson Model D Electrophorator. Areas corresponding to sulfur-containing compounds, located by reference to unlabeled standards stained with ninhydrin, were cut out, placed in vials containing scintillation fluid (Econofluor, New England Nuclear), and counted. The extent of labeling of each compound examined was expressed as a per cent of the original total counts per min spotted.

Assays for total intracellular glutathione were carried concurrently with the cystine analysis in separate dishes. Glutathione was determined by a modification (12) of the enzyme-cycling method of Owens and Belcher (13). For this purpose, cells washed three times with phosphate buffered saline were scraped directly into 0.02 N HCl.

For total amino acid analysis, confluent cells in duplicate 75-cm<sup>2</sup> flasks were harvested by trypsinization, washed three times with phosphate buffered saline, resuspended in 1 ml of 4% sulfosalicylic acid, sonicated for 20 s, and centrifuged for 10 min at 2000 rpm. The supernatant was subjected to amino acid analysis on a Beckman 121 M amino acid analyzer and the pellet was analyzed for protein by the method of Lowry *et al.* (11).

### RESULTS

The  $\gamma$ -glutamyl hydrazone of  $\alpha$ -ketobutyric acid, maleate, and serine in sodium borate buffer, known inhibitors of  $\gamma$ -

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† To whom reprint requests should be addressed.

§ Recipient of a Faculty Development Award from the Pharmaceutical Manufacturers Association Foundation and supported by a Basil O'Connor Research Starter Grant from the March of Dimes Birth Defects Foundations. Present address, Department of Pharmacology and Experimental Therapeutics, and Pediatrics, Johns Hopkins School of Medicine, Baltimore, MD 21205.

glutamyl transpeptidase (9, 14, 15), all produced dose-dependent decreases in the cystine content of cystinotic cells (Table I). As seen in this table, cystine content of untreated cystinotic skin fibroblasts was found to vary from 8 to 22 nmol of half-cystine/mg of cell protein, depending upon the particular cell line employed. However, each cell line had its own characteristic and reproducible cystine content when analyzed at confluency, which was always 50- to 100-fold greater than cystine accumulation in normal skin fibroblasts. Intracellular cystine was lowered 55% in 24 h by the higher concentration (20 mM) of inhibitors. Borate or serine alone was without effect. The time course of cellular depletion of cystine in the presence of serine-borate (40 mM) is shown in Fig. 1. Cystine content was decreased by 72% in 6 days, at which time cells appeared morphologically normal. After 6 days, many cells appeared rounded up but still remained attached to the surface.

To confirm the loss of intracellular cystine, cells which had been preincubated in medium containing 40 mM serine-borate for 24 h were then incubated in fresh media containing 40 mM serine-borate and L-[ $^{35}$ S]cystine (10  $\mu$ Ci/ml) for 24 h (Table II). Cystinotic cells took up an average of 60% more radioactivity than normal cells and the presence of serine-borate reduced this difference to 38%. In normal cells, 79% of this label was found in glutathione and 2% in cystine, while in

TABLE I  
Cystine content of cystinotic cells treated with  $\gamma$ -glutamyl transpeptidase inhibitors for 24 h

Confluent cystinotic skin fibroblasts in 60-mm dishes were incubated for 24 h in medium containing inhibitors of  $\gamma$ -glutamyl transpeptidase as indicated and analyzed for cystine by the cystine-binding protein assay of Oshima *et al.* (10) and for protein by the method of Lowry *et al.* (11). Cystine values represent the average of two to four samples. Each  $\gamma$ -glutamyl transpeptidase inhibitor was tested with a different cystinotic cell line resulting in different cystine levels in the untreated controls. Cell lines used were as follows: maleate, line D;  $\gamma$ -glutamyl hydrazone of  $\alpha$ -ketobutyric acid, line E; serine-borate, line F; borate alone, line G; serine alone, line H.

Inhibitor concentration	Maleate	$\gamma$ -Glutamyl hydrazone of $\alpha$ -ketobutyric acid	Serine-borate	Borate	Serine
mM					
0	22.1 <sup>a</sup> (100) <sup>b</sup>	10.44 (100)	10.03 (100)	15.8 (100)	8.82 (100)
1	18.3 (83)	8.74 (84)	7.21 (72)	17.5 (111)	8.23 (93)
5	14.8 (67)	6.10 (58)	5.91 (59)	17.7 (112)	
10	11.4 (52)	4.06 (39)	5.13 (51)	14.9 (94)	9.13 (103)
20	10.4 (47)	4.58 (44)	4.65 (46)	15.2 (96)	

<sup>a</sup> Nanomoles of half-cystine/mg of cell protein.

<sup>b</sup> Per cent of untreated control.

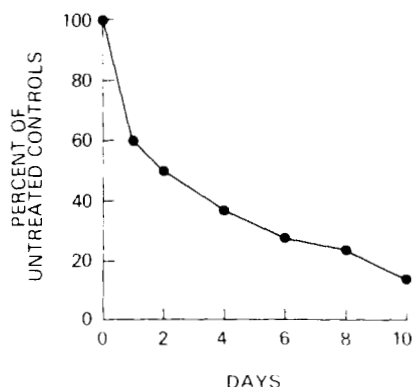


FIG. 1. Cystine content of confluent cystinotic fibroblasts treated with serine-borate (40 mM each) for 10 days. Cells were refed every 2nd day, harvested at the times indicated, and subjected to cystine analysis by the cystine-binding protein assay as described under "Materials and Methods."

TABLE II

Glutathione and cystine content of normal and cystinotic cells labeled with L-[ $^{35}$ S]cystine in the presence and absence of 40 mM serine-borate

Confluent normal and cystinotic skin fibroblasts in 60-mm dishes were used. Half of the dishes were preincubated for 24 h with medium containing serine-borate (40 mM each) followed by a further 24-h incubation in fresh medium containing serine-borate (40 mM each) and [ $^{35}$ S]cystine (10  $\mu$ Ci/ml). Control dishes were treated in the same manner, except for the addition of phosphate buffered saline instead of serine-borate. Cells were harvested and processed for detection of radioactive label in glutathione and cystine by high voltage electrophoresis as outlined under "Materials and Methods." SB, serine-borate; PBS, phosphate-buffered saline.

Cell line	Radioactivity		% of total counts <sup>a</sup> in			
	Control (+ PBS)	Experi- mental (+ SB)	Glutathione		Cystine	
			+ PBS	+ SB	+ PBS	+ SB
<i>cpm/mg cell protein</i>						
Normal						
A	1977	1844	87	93	1.2	0.8
B	1707	2072	74	92	2.7	1.7
C	1529	2219	75	82	2.1	1.2
Cystinotic						
D	2980	3042	44	65	45	28
E	2599	2627	56	64	35	28

<sup>a</sup> Values represent per cent of total applied radioactivity and are the average of duplicate analyses. Average counts per min spotted was 30,000.

TABLE III

Intracellular amino acid levels in normal and cystinotic skin fibroblasts cultured in the presence and absence of serine-borate (40 mM)

Confluent fibroblasts were incubated in the presence and absence of 40 mM serine-borate for 48 h. Cells were then harvested and prepared for amino acid analysis as outlined under "Materials and Methods."

Amino acids	Normal fibroblasts <sup>a</sup>		Cystinotic fibroblasts <sup>a</sup>	
	Control	Serine-borate	Control	Serine-borate
Threonine	16.6 $\pm$ 3.8 <sup>b</sup>	19.1 $\pm$ 5.4	10.8 $\pm$ 1.3	14.5 $\pm$ 1.4
Proline	21.4 $\pm$ 4.6	7.4 $\pm$ 1.7 <sup>c</sup>	12.3 $\pm$ 1.2	4.9 $\pm$ 0.2 <sup>c</sup>
Glutamic acid	150.2 $\pm$ 22.2	154.7 $\pm$ 28.2	96.8 $\pm$ 9.8	129.0 $\pm$ 6.5 <sup>c</sup>
Glycine	30.9 $\pm$ 8.9	20.3 $\pm$ 6.0	17.8 $\pm$ 2.3	15.4 $\pm$ 1.5
Alanine	27.2 $\pm$ 6.8	28.8 $\pm$ 8.7	16.5 $\pm$ 1.9	21.1 $\pm$ 1.9
Valine	7.4 $\pm$ 2.1	6.0 $\pm$ 1.2	6.6 $\pm$ 0.6	5.5 $\pm$ 0.3
Half-cystine	0.3 $\pm$ 0.2	0.6 $\pm$ 0.3	19.3 $\pm$ 2.3	9.9 $\pm$ 1.9 <sup>c</sup>
Methionine	2.6 $\pm$ 0.3	1.6 $\pm$ 0.4	2.2 $\pm$ 0.1	1.9 $\pm$ 0.1
Isoleucine	8.1 $\pm$ 1.5	5.8 $\pm$ 1.1	6.8 $\pm$ 0.5	6.5 $\pm$ 0.4
Leucine	12.7 $\pm$ 2.0	8.7 $\pm$ 1.6	9.2 $\pm$ 0.7	8.2 $\pm$ 0.3
Tyrosine	5.6 $\pm$ 1.0	3.8 $\pm$ 0.8	4.2 $\pm$ 0.3	3.6 $\pm$ 0.1
Phenylalanine	6.7 $\pm$ 0.5	4.4 $\pm$ 0.5	5.3 $\pm$ 0.3	4.6 $\pm$ 0.2
Lysine	9.7 $\pm$ 1.1	4.6 $\pm$ 1.8	7.6 $\pm$ 0.5	6.4 $\pm$ 0.3
Histidine	4.5 $\pm$ 0.9	2.4 $\pm$ 1.0	3.4 $\pm$ 0.2	3.0 $\pm$ 0.2
Tryptophan	6.4 $\pm$ 1.5	8.6 $\pm$ 0.2	7.2 $\pm$ 0.6	7.8 $\pm$ 1.1
Arginine	7.6 $\pm$ 0.8	3.8 $\pm$ 1.5	6.5 $\pm$ 0.5	5.3 $\pm$ 0.1

<sup>a</sup> Three cell lines of each were analyzed. Normals were lines A, B, and C; cystinotics were lines D, E, and G.

<sup>b</sup> Nanomoles of half-cystine/mg of cell protein  $\pm$  S.E.

<sup>c</sup> Serine-borate treatment resulting in significant differences from untreated controls ( $p < 0.05$ ).

cystinotic cells 50% of the label was located in glutathione and 40% in cystine. Both normal and cystinotic serine-borate-treated cells showed a decrease in per cent of total label incorporated into intracellular cystine and a small increase in glutathione when compared with phosphate buffered saline-treated controls.

To determine the effect of a  $\gamma$ -glutamyl transpeptidase inhibitor on cystine and glutathione levels in normal cells, fibroblasts were treated with serine-borate (40 mM each). The cystine content of normal cells measured by cystine-binding

protein, while much lower than that in cystinotics ( $0.25 \pm 0.06$  nmol of half-cystine/mg of cell protein), was further lowered a small amount by serine-borate treatment (mean reduction of cystine 13.8%,  $n = 6$ ). Furthermore, there was no significant difference in glutathione content of untreated cystinotic and normal cells ( $6.69 \pm 1.02$  versus  $6.06 \pm 1.50$   $\mu$ g of glutathione/mg of protein,  $n = 10$ ), and serine-borate treatment did not cause any significant changes in glutathione content of these cells as measured by enzyme cycling (12, 13).

The data in Table III, obtained by amino acid analysis, indicate that the levels of most amino acids were unchanged by serine-borate treatment of normal and cystinotic cells. Lower levels of glutamic acid and glycine (constituents of glutathione) are seen in untreated cystinotic cells as compared to normal cells. A significant increase toward normal levels was seen for glutamic acid when cystinotic cells were treated with serine-borate. The decrease in proline concentration seen in cystinotic cells and the further decreases caused by serine-borate treatment are not understood and are under further investigation. While cystine content of normal cells was unchanged, cystine content of cystinotic cells was decreased by one-half, a result consistent with our data for uptake of L-[ $^{35}$ S]cystine in the presence of serine-borate, and with cystine analysis by the cystine-binding protein assay of cells incubated in the presence of serine-borate and other  $\gamma$ -glutamyl transpeptidase inhibitors.

#### DISCUSSION

Most cells contain large amounts of glutathione which could serve as a source of accumulated cystine in cystinotic cells. Free cysteine in culture medium is toxic to tissue culture cells (16). Tateishi *et al.* (17) have proposed that glutathione serves as a pool of a nontoxic form of cysteine which is freed for cellular use by the turnover of glutathione in the  $\gamma$ -glutamyl cycle (7, 8). Orloff *et al.* (18) have shown that cystinotic cells are more resistant to the toxic effects of free cysteine than are normal cells. Patrick *et al.* (19) have reported that  $\gamma$ -glutamyl transpeptidase activity in normal and cystinotic cells does not differ when tested in broken cell preparations.  $\gamma$ -Glutamyl transpeptidase activity of normal and cystinotic cell lines maintained in our laboratory also did not differ significantly.<sup>1</sup> In contrast to these results, States and Segal (20) have recently reported a significant difference in normal and cystinotic  $\gamma$ -glutamyl transpeptidase activity in a large number of fibroblast lines. There is considerable overlap in their data, but such increased activity would allow for a more rapid turnover of glutathione in cystinotic than in normal cells.

Our studies show that interfering with glutathione catabolism by inhibition of  $\gamma$ -glutamyl transpeptidase will decrease the cystine content of cystinotic cells. Normal cells also show decreased cystine content after serine-borate treatment but to a much lesser degree. Several different inhibitors were effective in a dose-responsive manner. Maleate inhibits  $\gamma$ -glutamyl transpeptidase by binding to the cysteinyl-glycine site, thereby inhibiting formation of the enzyme-substrate complex and also by blocking binding of the acceptor amino acid (14). The  $\gamma$ -glutamyl hydrazones of the  $\alpha$ -keto acids inhibit  $\gamma$ -glutamyl transpeptidase by competition for the  $\gamma$ -glutamyl donor site (9, 21). Serine-borate forms a complex with  $\gamma$ -glutamyl transpeptidase at the  $\gamma$ -glutamyl-binding site and prevents the formation of the transition state (22). In our studies, borate alone had no effect on cystine storage, eliminating borate inhibition as a factor. While serine by itself inhibited  $\gamma$ -glutamyl transpeptidase to a minor extent (9), it did not

influence the level of cystine in cystinotic cells. The time course of cystine lowering by serine-borate treatment showed continued decreases for 10 days, although after 6 days cells manifested some signs of toxicity. At 48 h, other intracellular amino acids were not in general significantly affected by serine-borate treatment and cells appeared morphologically normal. Lowering of intracellular cystine by inhibition of  $\gamma$ -glutamyl transpeptidase activity was demonstrated by three methods of analysis, *viz.* cystine-binding protein assay, radioactive labeling, and automated amino acid analysis.

The mechanism for accumulation of cystine in cystinotic cells is uncertain. Our results suggest that turnover of glutathione may be important in regulating cellular cystine levels. The following possibilities for such regulation may be considered. First, inhibition of glutathione catabolism could provide increased glutathione to chemically reduce intracellular cystine. However, such an increase in intracellular glutathione content was not detected in either normal or cystinotic cells and, furthermore, glutathione content,  $\gamma$ -glutamyl transpeptidase activity, and glutathione reductase levels were found to be normal in cystinotic cells (19, 23). Griffith *et al.* (24) have shown that serine-borate given to mice results in increased levels of intracellular glutathione and a decrease in the rate of glutathione utilization. We did not see increased glutathione levels when normal and cystinotic fibroblasts were treated with serine-borate, however, when such cells were labeled with L-[ $^{35}$ S]cystine, more label appeared in the serine-borate treated cells, indicating a decreased turnover of glutathione. In glutathione synthetase-deficient fibroblasts which have 10% of normal glutathione content, serine-borate treatment more than doubled cell glutathione content (25).

Second,  $\gamma$ -glutamyl transpeptidase has been postulated to be involved in amino acid transport. Since cystine is the best amino acid acceptor for this enzyme (26), its transport in cystinotic cells could be affected by altered enzyme activity. This postulated mechanism for cystine transport may not be critical as demonstrated in transport experiments with cultured mutant cells which are markedly deficient in  $\gamma$ -glutamyl transpeptidase; such cells were found to transport cystine normally (27).

Third, abnormal formation of cystine may result from altered turnover of reduced or oxidized glutathione or glutathione-cysteine mixed disulfide. Our data on the incorporation of L-[ $^{35}$ S]cystine (Table II) show that inhibition of  $\gamma$ -glutamyl transpeptidase leads to increased labeling of glutathione and decreased label in cystine. Bannai and Tsukeda (28) have found that glutathione is normally secreted from diploid cells in culture and, in the presence of medium, cystine forms the glutathione-cysteine mixed disulfide. Since *S*-substituted glutathiones are good substrates for  $\gamma$ -glutamyl transpeptidase (9, 28), the catabolism of glutathione-cysteine by  $\gamma$ -glutamyl transpeptidase and its associated nonspecific membrane peptidase (7, 8) could provide readily available cellular cystine.

The demonstrated reduction of cystine levels in cystinotic cells by  $\gamma$ -glutamyl transpeptidase inhibitors suggests that altered transpeptidation may play a role in cystine accumulation in cystinosis. Regulation of  $\gamma$ -glutamyl transpeptidase activity is only partially understood.  $\gamma$ -Glutamyl transpeptidase is a membrane protein and its activity may be altered by its lipid environment (29) as has been shown for a number of other membrane proteins (30). Thus an altered membrane microenvironment might play a role in glutathione turnover and cystine accumulation.

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<sup>1</sup> J. DeB. Butler and S. P. Spielberg, unpublished data.

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