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# DEPLETION OF CYSTINE IN CYSTINOTIC FIBROBLASTS BY HOMOCYSTEINE

## SYNERGISM OF CYSTEAMINE WITH VARIOUS REDUCING AGENTS IN DEPLETION OF CYSTINE FROM CYSTINOTIC FIBROBLASTS

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**Abstract**—The present study shows that homocysteine depleted cystine from cystinotic fibroblasts *in vitro*. No toxic effects were noted as judged by morphology and growth patterns. Efflux of radioactivity from cystinotic cells prelabeled with [$^{35}$S]cystine was greater in homocysteine-treated cystinotic cells than in untreated controls. This radioactivity was found, by high voltage electrophoresis separation of effluxed products, to consist mainly of [$^{35}$S]cystine, along with smaller amounts of [$^{35}$S]homocysteine–cysteine mixed disulfide. When homocysteine and cysteamine were presented together to cystinotic cells at dose levels individually ineffective in removing cystine from these cells, a marked synergistic effect was observed and cystine content fell to 10% of that seen in untreated cystinotic fibroblasts. Similarly, synergistic effects of cystine depletion from cystinotic cells were demonstrated when cells were treated with a combination of cysteamine and dithiothreitol or glutathione. Incubation of cystinotic cells with homocysteine, dithiothreitol, or cysteamine in combination with vitamin C did not yield synergistic effects. The above findings suggest a novel way to probe metabolic processes in these mutant cells. Exploration of these synergistic effects may lead to more efficacious therapeutic protocols for cystinosis.

Cystinosis is an autosomal recessive human genetic disease where cystine is stored in many tissues at levels 50- to 100-fold of those seen in normal tissues [1]. Nephropathic cystinosis is the most severe form of this disease. Patients in the first years of life present with failure to thrive, development of Fanconi syndrome and subsequent kidney failure by 10 years of age. The stored cystine is confined to lysosomes [1, 2]. The cause of cystinosis is thought to be the lack of an as yet uncharacterized lysosomal membrane transport system for cystine [3]. Hence, treatment is aimed at depleting cystine stores. Patients are given cysteamine which rapidly depletes intracellular cystine to relatively low levels [4–6]. The mechanism of action of cystine depletion by cysteamine is believed to be one of thiol disulfide exchange where newly formed cysteamine–cysteine mixed disulfide can egress from lysosomes [7], and from whole cells [8]. One other seemingly unrelated compound, vitamin C (ascorbic acid), is also known to lower cystine levels in cystinotic fibroblasts [9]. Its mechanism of action is unknown as yet, but like cysteamine it can act as a reducing agent and helps maintain normal oxidative-reductive homeostasis of cells [10]. Vitamin C was given to cystinotic children in an extensive but unsuccessful therapeutic trial which was terminated after 2 years [11]. It should be pointed out, however, that the metabolism of vitamin C by cystinotic patients was not studied in that protocol. Vitamin C binds to dithiols but will not reduce cystine [12]. Vitamin C readily auto-oxidizes but may be kept in reduced form with agents such as homocysteine and dithiothreitol [13, 14]. The initial purpose of the present investigation was to ascertain if cell cystine content was alterable with homocysteine, and by what methods. In the course of the work, synergistic effects between cysteamine and other reducing agents were discovered, investigation of which became primary in this report.

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### MATERIALS AND METHODS

Skin fibroblasts were obtained from nephropathic cystinotic patients (after informed consent) and grown in tissue culture using standard techniques. Cell lines from four patients were used. Cells were plated on 35-mm diameter 6 well plates (Costar, Data Packaging, Cambridge, MA) incubated at 37° in 5% $CO_2$ and 95% air in Eagle's Minimum Essential Medium (NIH Media Preparation Department) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY) and 2 mM glutamine renewed twice weekly. No antibiotics were used, and tests for mycoplasma were negative. Fibroblasts have an absolute requirement for cystine [15].

For the studies on depletion of intracellular cystine, triplicate wells of cells were grown to confluency, treated with the various agents, harvested by trypsinization [0.25% trypsin in phosphate-buffered saline (PBS)] and prepared for cystine analysis by the cystine binding protein assay of Oshima *et al.* [16]. Total protein was determined by the method of Lowry *et al.* [17].

For study of cystine depletion and synergistic effects of various reducing agents, confluent cells

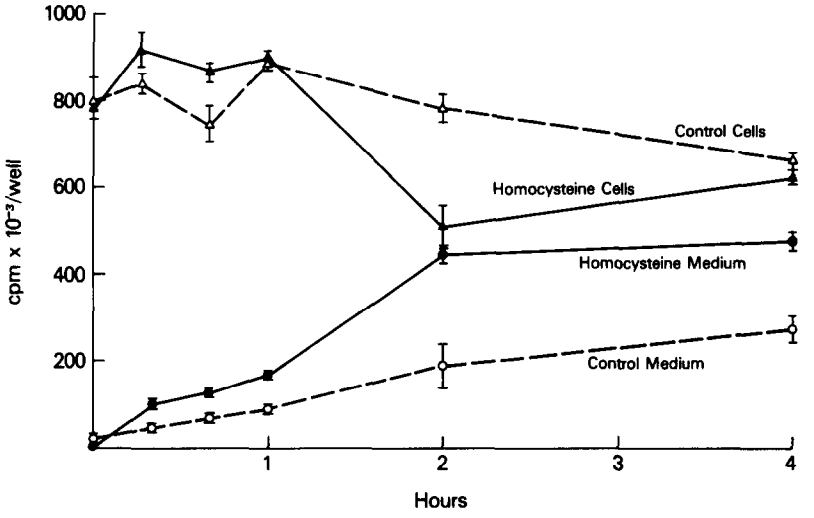


Fig. 1. Efflux of [$^{35}$S]cystine from cystinotic fibroblasts prelabeled for 24 hr, and then incubated in medium with an without 10 mM homocysteine. At the indicated times, medium and cells were harvested and deproteinated, and radioactive label in medium and cells was counted as outlined in Materials and Methods.

were incubated with medium containing D,L-homocysteine, L-homocystine (Fluka Chemical Corp., Ronkonkoma, NY), cysteamine, dithiothreitol, glutathione, and vitamin C (Sigma Chemical Co., St. Louis, MO) and various combinations for the time periods and concentrations indicated in Results. Cells were harvested and processed as outlined above for cystine depletion studies.

For radioactive labeling studies, confluent cystinotic cells were washed twice with experimental medium (cystine free minimum essential medium containing 2 mM glutamine and 1 mg/mL bovine serum albumin in place of serum), and then incubated with [$^{35}$S]cystine (New England Nuclear, Boston, MA) for 24 hr in experimental medium that contained 10 $\mu$Ci/mL [$^{35}$S]cystine. Extracellular radioactivity was then removed by washing the cells five times with experimental medium. These plated cells containing [$^{35}$S]cystine and its metabolites were incubated for various times in 1.5 mL of experimental medium with or without 10 mM homocysteine. Addition of homocysteine defined zero time, and 3 wells were used for each point. Since analysis of confluent replicate wells yielded equivalent protein concentrations, the data are expressed as cpm/well. At 20 min and 2 hr, the distribution of non-protein radioactivity among subcellular fractions and extracellular medium was determined. At harvest time, 1 mL of medium was removed and protein immediately precipitated by addition of 0.1 mL of 30% sulfosalicylic acid (Sigma) and placed on ice. Cells were washed twice with PBS, trypsinized in 1 mL of 0.25% trypsin in PBS, centrifuged at low speed (600 $g$) and washed three times with PBS to remove trypsin. Cells were resuspended in 1 mL of 0.25 M sucrose, sonicated for 10 sec with a microprobe sonicator (Heat-Systems Ultrasonics, Inc., Plainview, NY) and centrifuged at 1000 $g$ for 10 min to produce a rough nuclear pellet. The supernatant fraction was centrifuged at 20,000 $g$ for 30 min yielding a granular pellet and a supernatant. The two pellets were resuspended in 0.9 mL water, and 0.1 mL bovine serum albumin (1 mg/mL) was added to each fraction and to the supernatant fraction as carrier; sulfosalicylic acid (0.1 mL, 30%) was added to precipitate proteins. All procedures after trypsinization were carried out at 4° without delay. Aliquots of the protein-free fractions were spotted on 3MM Whatman chromatography paper (Whatman Laboratory Products, Inc., Whatman Paper Division, Clifton, NJ), and [$^{35}$S]cystine-labeled products were separated in a High Voltage Electrophorator, model D, Gilson Medical Electronics (Middleton, WI) at 4 kV for 1 hr in 7.4% formic acid. Standards were run on each paper and stained with ninhydrin (0.25%, in acetone) to locate the products. The paper chromatograms were cut in 1 cm strips and counted in Aquasol (New England Nuclear, Boston, MA) in a Beckman LS-250 scintillation counter.

## RESULTS

D,L-Homocysteine, at a concentration of 10 mM, depleted cystinotic fibroblasts of cystine (Table 1), lowering the intracellular cystine concentration to 10% of cystine levels in untreated control cystinotic cells in 8 hr; this level was maintained for at least 24 hr. Reaccumulation of cystine occurred when cells were incubated in homocysteine-free medium (data not shown). At the lower concentration of 1 mM D,L-homocysteine, cystine levels decreased much more slowly, arriving at 57% of control values by 24 hr. D,L-Homocysteine at 10 mM was not toxic to the cells as judged by morphology, trypan blue dye exclusion test, and continued growth of treated cells (data not

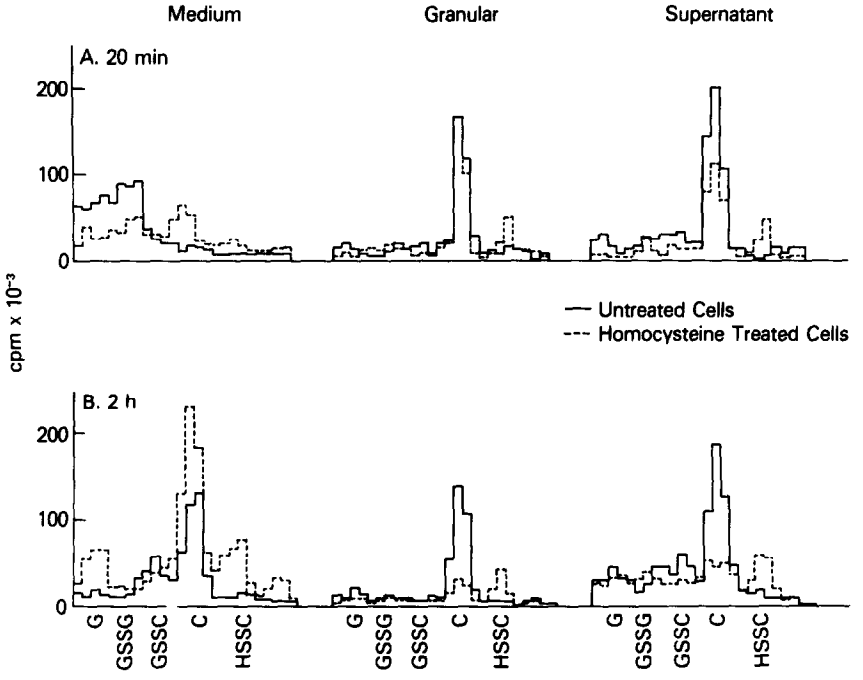


Fig. 2. Identification of [$^{35}$S]cystine products in medium and in granular and supernatant cell fractions. Confluent cystinotic cells prelabeled for 24 hr with [$^{35}$S]cystine were washed clean of radioactivity and allowed to efflux into fresh medium with and without homocysteine. At 20 min and 2 hr cells were harvested, washed, resuspended in 0.25 M sucrose, homogenized and separated into three crude fractions by centrifugation; radioactive products were identified by high voltage electrophoresis as outlined in Materials and Methods. Paper strips (1 cm) were counted for radioactivity and matched with controls run on the same chromatogram to identify the products. Abbreviations are as follows: glutathione (G), oxidized glutathione (GSSG), glutathione–cysteine mixed disulfide (GSSC), cystine (C), and homocysteine–cysteine mixed disulfide (HSSC).

Table 1. Depletion of cystine in cystinotic fibroblasts by D,L-homocysteine and L-homocystine

| Treatment | Concn (mM) | Incubation time (hr) | (nmol ½ Cystine/mg cell protein) |
|---|---|---|---|
| None | | 0 | 16.4 ± 1.0 |
| D,L-Homocysteine | 10 | 0.5 | 9.6 ±0.4 |
| | | 1 | 7.5 ± 1.0 |
| | | 2 | 3.6 ± 0.2 |
| | | 4 | 2.0 ± 0.1 |
| | | 8 | 1.4 ± 0.1 |
| | | 24 | 1.6 ± 0.2 |
| | 1 | 2 | 15.8 ± 0.8 |
| | | 4 | 15.4 ± 0.3 |
| | | 8 | 12.8 ± 1.1 |
| | | 24 | 9.4 ± 1.1 |
| L-Homocystine | 1 | 0 | 23.3 ± 1.4 |
| | | 4 | 20.6 ± 1.2 |
| | | 24 | 17.9 ± 1.7 |

Confluent cystinotic fibroblasts were incubated at 37°, 5% $CO_2$ atmosphere in minimum essential medium, 10% fetal bovine serum containing the above concentrations of D,L-homocysteine or L-homocystine, harvested at the indicated times, and analyzed for cystine and protein content as outlined in Materials and Methods. Values are means ± SD, N = 3.

Table 2. Depletion of cystine and cystinotic fibroblasts by homocysteine and vitamin C

| Treatment | Concn. (mM) | (nmol ½ Cystine/mg cell protein) |
|---|---|---|
| None | | 14.7 ± 0.9 |
| Vitamin C | 0.5 | 8.9 ± 0.3 |
| Homocysteine | 0.5 | 9.0 ± 1.4 |
| Vitamin C and homocysteine | 0.5<br>0.5 | 6.0 ± 0.8 |

Confluent cystinotic fibroblasts were incubated at 37°, 5% $CO_2$ atmosphere in minimum essential medium containing the above concentrations of vitamin C and D,L-homocysteine, harvested at 2 days, and analyzed for cystine and protein content as outlined in Materials and Methods. Values are means ± SD, N = 3.

shown). Homocystine, the oxidized form of homocysteine, was less effective, depleting cystinotic intracellular cystine by 23% in 24 hr. Note that each cystinotic cell line has a characteristic steady-state level of accumulated cystine at confluency, accounting for the difference in initial (zero time) cystine levels in the homocysteine and homocystine experiments (Table 1).

When cystinotic fibroblasts were prelabeled with [$^{35}$S]cystine and subsequently incubated with fresh non-radioactive medium with and without D,L-homocysteine, radioactive products effluxed to the medium of the homocysteine-treated cells at a rate approximately twice that seen in control untreated cells at all times tested (Fig. 1). At 2 hr, intracellular radioactivity of treated cells was lower than that of untreated control cells, while at the early times of 20 and 40 min, radioactive products were higher in treated cells. By 4 hr of incubation, these differences between radioactive products in homocysteine-treated and untreated cystinotic cells essentially disappeared, whereas products effluxed to the medium were still 1.6 times higher when homocysteine was used. Similar accumulation of radioactivity in treated and non-treated cells at 4 hr may reflect reuse of radioactive products released to the medium by the treated cells.

These cells were then fractionated in 0.25 M sucrose as previously outlined, yielding three crude fractions: nuclear and granular pellets, and supernatant. Again, radioactivity in the granular pellet and in the supernatant fraction of homocysteine-treated cells was less at 2 hr from that seen in non-treated control cells, while the increased radioactivity seen in homocysteine-treated (10 mM) whole cells at early times of 20 and 40 min (Fig. 1) was found in the cytosol fraction (data not shown). Table 1 shows that cystine was depleted by homocysteine as early as 30 min, while cell separation studies showed that radioactive cystine products were removed from the cell more slowly. Note that when cells were prelabeled for 24 hr with [$^{35}$S]cystine, label was distributed to many compartments of the cell. Thus, small differences in [$^{35}$S]cystine label seen in homocysteine, treated cells at 4 hr in Fig. 1 will not correspond to large differences in cell cystine content seen at 4 hr in Table 1 where cellular cystine was measured by the specific cystine binding protein assay [16]. The specificity of the cystine binding protein assay was tested by including homocystine, homocysteine and a mixture of homocysteine and cysteamine in the assay. No interference in cystine quantitation was detectable.

To determine the products of [$^{35}$S]cystine metabolism by cells treated with D,L-homocysteine and allowed to efflux into fresh medium, chosen aliquots of deproteinated media, and granular and supernatant fractions were separated by high voltage electrophoresis as outlined in Materials and Methods. In Fig. 2, panel A, at 20 min, medium from homocysteine-treated cells shows efflux of cystine and of glutathione products, while medium from untreated control cells shows efflux of glutathiones and essentially no cystine. The 20-min supernatant fraction of homocysteine-treated cells shows a corresponding fall in cystine content and formation of a small amount of homocysteine–cysteine mixed disulfide, while the granular fraction shows little change in cystine content and again a small amount of homocysteine–cysteine mixed disulfide. As shown in panel B of Fig. 2, at 2 hr, the major efflux of radioactive material from the cell to the medium was cystine. In the granular and supernatant fractions of the homocysteine-treated cells, very little cystine remained as compared to that seen in untreated cells. Thus, in treated cells cystine seemed to be removed first from the cytosol and later from the granular or lysosomal fraction, appearing in medium predominantly as cystine. In untreated cells, cystine first effluxed as glutathiones and later as cystine.

To better understand the mechanism of depletion of intracellular cystine, confluent cystinotic cells were then incubated for 4 hr with various mixed concentrations of D,L-homocysteine and cysteamine. Figure 3 illustrates a notable synergistic effect of the combined drugs. At a dose level of 0.02 mM cysteamine and 1 mM homocysteine where no depletion of cystine in cystinotic cells was apparent when these compounds were used individually, the combined drugs lowered cystine levels by 88% as compared to controls. At a dose of 0.03 mM cysteamine and 1.5 mM homocysteine where the theoretical combined effect on cystine would be 40% depletion, the experimental depletion was 90%. Even at the low dose levels of 0.01 mM cysteamine and 0.5 mM homocysteine, an impressive 75% reduction of cystine levels was obtained.

The data were suggestive that other thiol reducing agents may have similar synergistic effects on lowering intracellular cystinotic cystine levels. Thus, mixing dithiothreitol with cysteamine gave a strong synergistic effect (Fig. 3). Also, combinations of glutathione and cysteamine were strongly synergistic. One millimolar glutathione (which increased cystine levels from 22 ± 1 to 27 ± 1 nmol ½ cystine/mg protein) with 0.02 mM cysteamine (which had no effect on cystine levels) lowered cystine levels to 2.8 ± 0.2 nmol ½ cystine/mg protein.

Homocysteine and dithiothreitol are known to keep vitamin C in the reduced state [13, 14], and vitamin C can deplete cystinotic cells of cystine stores [9]; thus experiments were performed with cystinotic

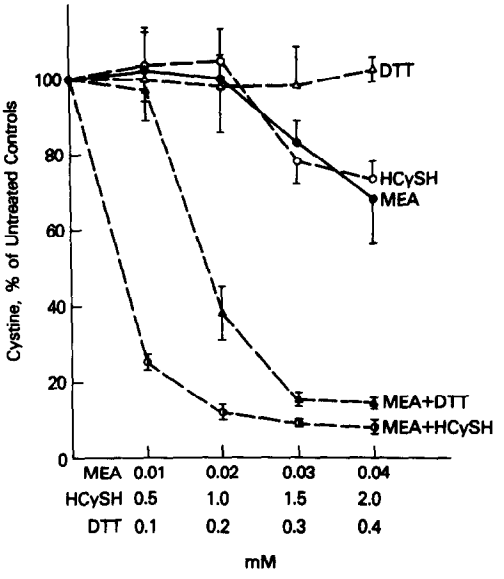


Fig. 3. Synergistic effect of cysteamine with homocysteine or dithiothreitol on cystine levels in cystinotic fibroblasts. Confluent cystinotic fibroblasts were incubated at 37°, 5% $CO_2$ atmosphere in minimum essential medium, 10% fetal bovine serum with and without cysteamine (MEA), homocysteine (HCSH), dithiothreitol (DTT) and combinations in the concentrations indicated. At 4 hr, cells were harvested and assayed for cystine and protein content as outlined in Materials and Methods. Results are plotted as per cent cystine of untreated cystinotic controls. Points were obtained from one to four separate experiments using four different cystinotic cell lines. Each experiment was carried out in triplicate. Absolute values for untreated controls ranged from 10 to 27 nmol ½ cystine/mg cell protein. The lowest values for the synergistic effect ranged from 1 to 3 nmol ½ cystine/mg cell protein. Normal fibroblasts contain about 0.2 nmol ½ cystine/mg cell protein.

cells using combinations of vitamin C with cysteamine, homocysteine or dithiothreitol. At 4 hr, these combinations of drugs failed to lower cystine any further than when these agents were used individually (data not shown). Since vitamin C is only effective in lowering cystine levels in cystinotic cells on a longer time scale, cells were incubated for 2 days with 0.5 mM vitamin C and 0.5 mM D,L-homocysteine (Table 2). Fresh medium was added at 24 hr. Homocysteine and vitamin C each lowered cystine levels 41%. The combination of homocysteine and vitamin C depleted cystine levels 60%. This effect was not synergistic but somewhat additive.

## DISCUSSION

The data presented here show that D,L-homocysteine can deplete cystinotic fibroblasts of their excess cystine. The major portion of cystine removed from cells treated with homocysteine appeared in the medium as free cystine and minor portions were seen as glutathiones and homocysteine–cysteine mixed disulfide. This is in contrast to that found in material effluxed from cystamine (oxidized cysteamine) treated cystinotic cells where cystine appears in the medium almost exclusively as the mixed disulfide cysteamine–cysteine [8]. In the case of incubation with homocysteine, it is possible that the exiting homocysteine–cysteine undergoes thiol disulfide exchange with medium homocysteine, releasing free cysteine which auto-oxidizes rapidly to form cystine. In the case of incubation with cystamine, such a thiol disulfide exchange is impossible.

Cysteamine, the drug of choice for treating patients, is thought to deplete cystinotic cells of cystine by thiol disulfide exchange forming the mixed disulfide cysteamine–cysteine which then can egress from lysosomes by a lysine egress pathway [7, 8, 18]. Several other thiol-containing compounds, WR-1065 and WR-2721 [19], pantethine [20], dithiothreitol [21] and dimercaprol [22], are capable of lowering cystine in cystinotic cells. WR-1065, WR-2721, and pantethine are converted to cysteamine in cells and thus can form cysteamine–cysteine mixed disulfide which egresses from the lysosome and the cell, but how the other thiols cause egress of cysteine is unknown. However, simple thiols such as ethane or propane thiols or β-mercaptoethanol which might be expected to enter cells and participate in formation of mixed disulfides are ineffective in lowering cystine in cystinotic cells [4], indicating that a mechanism other then thiol disulfide exchange may be involved.

The thiol peptide glutathione seems to be involved in cystine depletion in cystinotic cells. Interference in the catabolism of cellular glutathione lowers cystinotic cellular cystine at least 50% [23]. Also during early growth of cystinotic cells, glutathione synthetase is twice as active as that seen in early growth of normal fibroblasts. In this same time frame, cystine levels in cystinotic cells fall to near normal concentrations [24]. However, glutathione and particularly glutathione–cysteine mixed disulfide are known to increase cystine levels in fibroblasts [25]. Regardless of the above effects, a synergistic lowering of cystinotic cystine was demonstrated when glutathione was combined with cysteamine in the cell medium. Since glutathione does not penetrate cells [26], this synergistic effect may occur outside the cell.

As noted in the introduction, cystine in cystinotic cells can be depleted by addition of excess ascorbic acid [9]. The maximum cystine lowering was about 50% in 2–3 days of daily treatment. The reason for this effect is as yet unknown. When ascorbic acid is incorporated into cells via liposomes, the depletion effect is greater and cystine is lowered to 25% of controls in 2 hr [27]. Thus, proper entry of ascorbic acid into cells may be of importance. In the 2-day experiment where homocysteine was combined with vitamin C (Table 2), cystine levels were lower than when the drugs were present alone in the medium. Homocysteine may contribute to this additive effect on cystine depletion by maintaining higher levels of reduced ascorbic acid in addition to its demonstrated ability to participate in thiol-disulfide exchange with the stored cystine.

Free radicals are known to be produced during normal cellular metabolic processes. These potentially lethal molecules are handled naturally by enzymatic and chemical reducing agents, such as

glutathione peroxidase, superoxide dismutase, catalase, glutathione, vitamins C and E, free cysteine and cysteines within peptides or proteins [28]. A defect in one element for balancing the oxidative-reductive state of a cell would cause the cell to rely more heavily on other components of the system. Free radicals can abstract hydrogens from other molecules rendering them nonfunctional and subject to autophagocytosis and degradation in lysosomes [29]. Cystine in cystinotic lysosomes has been suggested to be derived from degradation of proteins in this organelle [30]. Thus, cystine could accumulate in cells that use alternative pathways for achieving a normal oxidative-reductive homeostasis.

In the present work, homocysteine, dithiothreitol and glutathione displayed strong synergism for cystine depletion when present in medium of cystinotic fibroblasts along with cysteamine. If this depletion were due only to thiol-disulfide exchange with cystine, results would only be additive. The observed synergism may indicate that two mechanisms of cystine depletion were operating. One of these has been well demonstrated to be thiol disulfide interchange [7, 8, 18]. The other has at least the following possibilities. First, the additional reducing agent along with cysteamine could be reducing the cysteamine–cysteine mixed disulfide as it egresses from the cell or the lysosome so that cysteamine is freed to initiate another round of cystine depletion. What happens to the freed cysteine is problematical. Glutathione–cysteine mixed disulfide increases cellular cystine [25], and free cysteine readily auto-oxidizes to cystine which in theory would exert pressure toward increasing intracellular cystine; neither effect was detected in these experiments. Second, the additional reducing agent may be furnishing electron equivalents needed by cystinotic cells to maintain a normal oxidative-reductive state relieving the pressure for use of cysteine and cysteine containing peptides and proteins as free radical scavengers. Third, these reducing agents may be maintaining a high level of intracellular vitamin C as they are known to do in the test tube [13, 14]. Non-observance of a synergistic cystine lowering effect of homocysteine with vitamin C may support this theory in that the combination of drugs had only semi-additive effects. A marginal lack of ascorbic acid (partially corrected by excess feeding of ascorbic acid [9] or by presentation of ascorbic acid via liposomes [24]) could be detrimental to many metabolic steps including enzymatic hydroxylations.

The synergism seen here when incubating cystinotic cells with cysteamine together with other reducing agents may prove to be a very useful tool in the investigation of cystine cellular metabolism and in the development of more effective methods to deplete cystine in these mutant cells. Cysteamine, currently used in treatment of nephropathic cystinotic patients (as well as radiation exposure victims), is decidedly toxic [31] (as is homocysteine or dithiothreitol), and dosages must be carefully adjusted to tolerable levels for each child. The dramatic synergistic effect of reducing agents used in combination with cysteamine *in vitro*, as uncovered in this work, may be useful in developing clinical protocols to treat such patients.

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