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Cystinotic Fibroblasts Are Depleted of Free-Cystine by Acid pH Medium

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Summary

The free (nonprotein) cystine content of human cystinotic fibroblasts was found to vary with the pH of their culture medium. Intracellular cystine content was highest at alkaline medium pH's and lowest at acidic medium pH's. When maintained in medium of pH 6.3, cystinotic fibroblasts lost 50% of their free-cystine content in 6 hr and over 95% in 30 hr. Cystine was the only amino acid to show significant variation with changes in the pH of the culture medium.

Children with nephropathic cystinosis develop several renal tubular malfunction during the first year of life and suffer from end-stage renal failure by 10 years age (11, 12). This autosomal recessive disorder of metabolism, which is expressed in fibroblasts, is characterized biochemically by marked elevations of intracellular free (nonprotein) cystine content. While the biologic defect resulting in the accumulation of cystine has not been described, it is known that the increased cystine in these cells is localized in lysosomes and is at least partially derived from protein degradation (14). Chloroquine, which accumulates in lysosomes leading to an increased lysosomal pH (3, 5, 9, 15) and the inhibition of lysosomal metabolism, also inhibits the accumulation of free-cystine by cystinotic fibroblasts (14). Since it is known that changes in extracellular pH may also affect lysosomal function (4, 6), we decided to study the effect of extracellular pH on the cystine content of cultured cystinotic fibroblasts.

MATERIALS AND METHODS

FIBROBLAST CULTURE

Human fibroblasts obtained from skin biopsies of two children and a fetus with cystinosis were maintained and assayed for cystine and protein as previously described (8) except that the cells were grown in closed Falcon flasks (75 cm^2). Unless otherwise stated, experiments were done using modified F-12 medium (2) supplemented with 10% fetal bovine serum. Before experiments, the pH was adjusted by changing the P_{CO_2} or, to obtain higher pH's, by adding small amounts of 1 N NaOH. Experiments were also performed using Eagle's minimal essential medium with 25 mM Hepes buffer and 10% fetal bovine serum. The pH of this medium was adjusted with 1 N HCl or 1 N NaOH and used with an atmosphere of room air. After incubation, each flask was opened and a sample of the medium was placed into a tube containing mineral oil. The pH was immediately determined by placing a pH electrode through the upper layer of oil and into the growth medium (10).

PROTEIN DEGRADATION

Protein degradation studies were performed according to the method of Bradley *et al.* (1) The long-lived, stable cellular proteins were labeled by incubating confluent cells in L-[1-^{14}C]-leucine (0.25 μCi/ml, New England Nuclear Co.) for 48 hr. The short-lived proteins were labeled with L-[4,5-^{3}H(N)]-leucine (40 μCi/ml, New England Nuclear Co.) which was added to the culture medium and the cells incubated for 30 min. Isotope-containing medium was removed and the cells washed rapidly four times with serum free medium followed by the addition of 3 ml of fresh medium of the appropriate pH, supplemented with 1% fetal bovine serum. At each time point, the medium was removed and both the pH and the trichloroacetic acid (5%, w/v)-soluble radioactivity were measured. Three milliliters of fresh medium of the appropriate pH was immediately added for the continuation of the incubation. The total radioactivity of the system was determined for each flask as described (1).

INTRACELLULAR AMINO ACIDS

Cells were grown in flasks at different pH's as described. After 24 hr, cells were harvested by scraping with a rubber policeman and the cellular pellet collected by centrifugation for 4 min at 1000 × *g*. The medium was withdrawn and one microcurie of [6,6(n)-^{3}H]sucrose (3 Ci/mmole, Amersham Co.) was added to 3 ml of the medium. The cells were resuspended in the labeled medium by gentle mixing and then centrifuged again. The labeled medium was removed and a portion acid precipitated with 12% sulfosalicylic acid. Forty-five microliters of 5 mM N-ethyl-maleimide were added to the cell pellet which was then broken by sonification (Kontes Cell Disrupter). Cellular protein was precipitated by the addition of 15 μl of 12% sulfosalicylic acid. Aliquots of the cellular and medium supernatants were counted in Aquasol (New England Nuclear Co.). The remaining supernatants were analyzed for amino acid content using a Durram D-500 automated amino acid analyzer. The amount of medium remaining in the cellular supernatant was determined based on the amount of radiolabel in the supernatant. The levels of amino acids determined to be in this amount of medium were subtacted from the cellular supernatant levels, yielding values for intracellular amino acids. The cellular protein was estimated using the procedure of Lowry *et al.* (7).

RESULTS

Cystinotic fibroblasts were depleted of their cystine content by treatment with 1 mM mercaptoethylamine (13) and then incubated for 48 hr in media ranging in pH from 6.7 to 8.1. The amount of cystine reaccumulated by the cells varied directly with pH, being lowest at acid pH and highest at basic pH (Fig. 1*A*). Cystine values varied in a similar manner (Fig. 1*B*) when the cells were placed in media of different pH for 24 hr without prior depletion of their cystine content. Figures. 1*A* and *B* were obtained with a bicarbonate buffering system. Similar results were obtained using Hepes buffer. When maintained in medium of pH 6.3, cystinotic fibroblasts lost 50% of their cystine content in approximately 6 hr and over 95% in 30 hr (Fig. 2). Cell viability, when studied by either

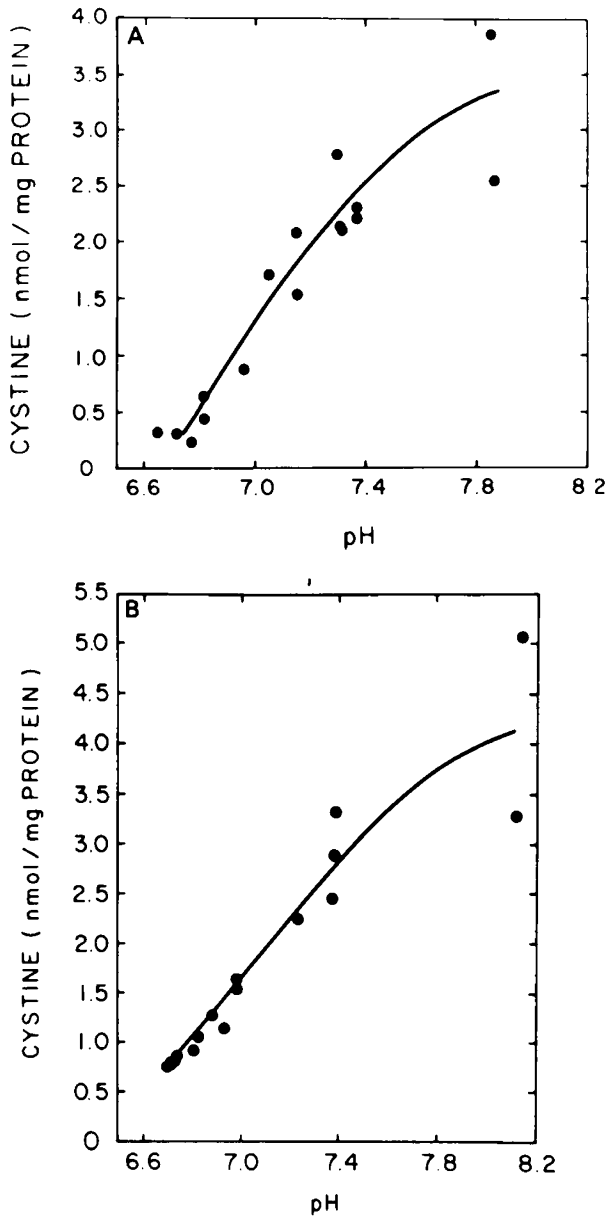

Fig. 1. The effect of media pH on the free-cystine content of cultured cystinotic fibroblasts at confluency. Cystine values were determined by a binding protein assay ("Materials and Methods"). Cystinotic cells were either treated with cysteamine before incubation at various pH's (*A*) or were placed directly into media of different pH's (*B*). Each value represents an individual flask of cells.

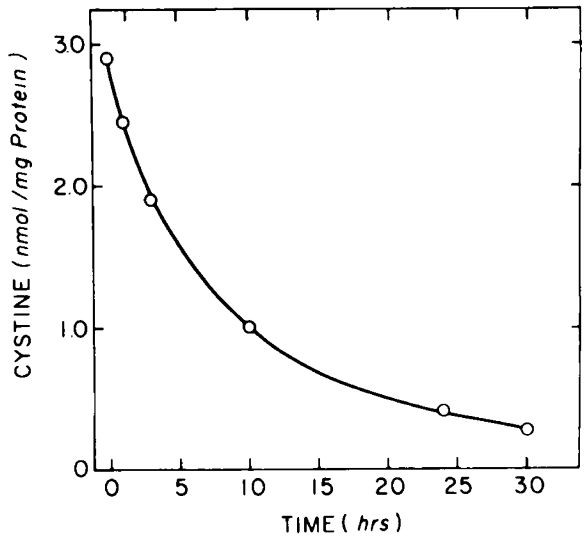

Fig. 2. The effect of acid pH (6.3) on the free-cystine content of cystinotic cells. Each point is the average of duplicate flasks. The media pH measurements were all within 0.1 units of the described value (6.3 ± 0.02, mean ± S.E.).

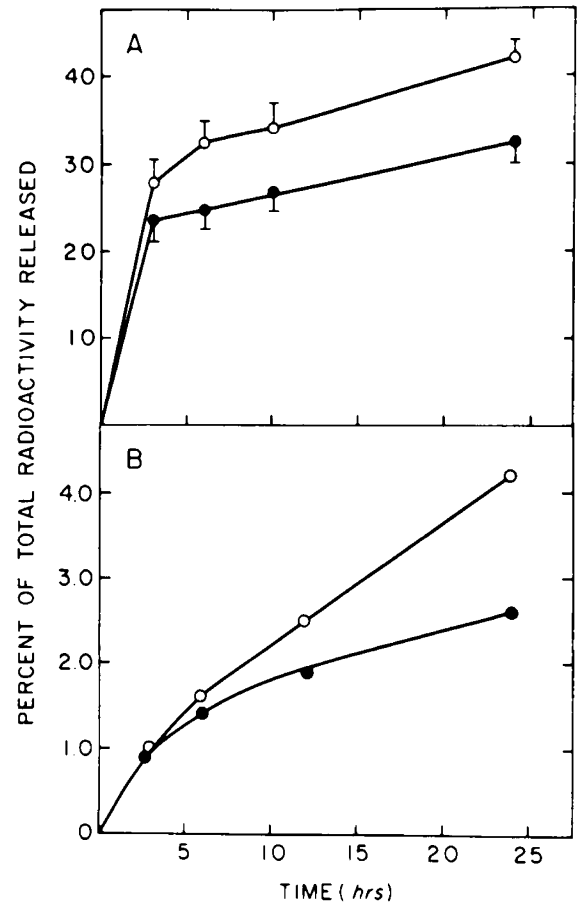

Fig. 3. The effect of media pH on the degradation of rapidly (*A*) and slowly (*B*) turning over intracellular proteins. Confluent monolayers of cystinotic fibroblasts in 25-cm^2 Falcon flasks were incubated at 37° with isotope as described in "Materials and Methods". In *A*, pH 7.4 medium (○) and pH 6.8 medium (●) were added. In *B*, pH 7.1 medium (○) and pH 6.4 medium (●) were added. Each *point* is the average of triplicate plates (*A*) or duplicate plates (*B*).

trypan blue dye exclusion or ^{14}C-amino acid incorporation into protein, was not affected by the extremes of pH used in this investigation. Surprisingly, the appearance of the cells by phase microscopy was also not remarkable at these pH's. The uptake of ^{35}S-cystine by these fibroblasts was not affected by the range of pH's described (data not shown).

Protein degradation studies showed that the degradation of both rapidly (*A*) and slowly (*B*) turning over proteins was inhibited when cells were placed in acidic medium (Fig. 3). The degradation of rapidly turning over proteins is inhibited by a constant percentage once the cells have been in acidic medium for 6 hr. The degradation of slowly turning over proteins is inhibited by an ever increasing percentage during the time that the cells are in acidic medium.

The levels of intracellular amino acids determined in cystinotic fibroblasts grown for 24 hr in minimal essential medium with Hepes buffer at different pH's are shown in Table 1. The data presented are based on three determinations of cellular amino acids at each medium pH. Cystine is the only amino acid which shows significant variation with the change in medium pH. Aspartic acid could not be measured in this study due to interference by the N-ethylmaleimide adduct of glutathione.

Table 1. *Amino acid concentrations in cystinotic fibroblasts after incubation for 24 hr at different pH's*

Amino acid:	pH 6.5 (nmole/ mg protein)	pH 7.5 (mean ± S.D.)	*P* (Student's *t* test)
Threonine	5.3 ± 0.6	8.0 ± 2.4	N.S.[2]
Serine	1.9 ± 0.7	1.4 ± 0.3	N.S.
Glutamic(ine)[1]	40.2 ± 11.4	45.6 ± 5.4	N.S.
Glycine	1.8 ± 0.2	2.5 ± 0.9	N.S.
Alanine	3.4 ± 0.4	5.5 ± 1.7	N.S.
Valine	5.3 ± 0.8	5.6 ± 0.8	N.S.
Cystine	4.7 ± 0.5	7.6 ± 0.8	<0.01
Methionine	1.0 ± 0.2	1.2 ± 0.6	N.S.
Isoleucine	4.6 ± 0.9	4.4 ± 0.3	N.S.
Leucine	5.5 ± 1.6	5.1 ± 1.0	N.S.
Tyrosine	2.6 ± 0.2	2.8 ± 0.6	N.S.
Phenylalamine	2.0 ± 0.2	2.3 ± 1.0	N.S.
Lysine	5.8 ± 1.8	6.4 ± 3.2	N.S.
Histidine	2.7 ± 2.4	3.4 ± 1.5	N.S.
Arginine	8.1 ± 1.1	6.6 ± 2.4	N.S.

[1] Results for glutamine and glutamic acid are combined.
[2] Nonsignificant ($P > 0.1$).

DISCUSSION

The lysosomal degradation of protein has been shown to be an important source of the cystine found in cultured cystinotic fibroblasts (14). The decreased turnover of both long- and short-lived intracellular proteins at lowered medium pH is consistent then with the finding that, under the same conditions, the cystine content of cystinotic fibroblasts is lowered. Not only is a lesser amount of cystine reaccumulated by cells in medium of acid pH, but cells actually lose cystine when the pH of their medium is lowered. This loss of cystine from cells at low medium pH is presumably secondary to a pH dependent equilibrium between the lysosomal efflux and deposition of cystine. The wide range of intracellular cystine content observed at varying pH levels of extracellular growth media occurred while the cystine content of the medium remained constant at 133 μM.

Cystine is the only cellular amino acid to vary significantly with medium pH. The isolated effect of pH on cystine in cystinotic cells may be a reflection of the localization of cystine in lysosomes. Other amino acids that are more widely distributed are apparently not as affected by medium pH. The variation of cystine concentration with pH does not seem to be related to a generalized loss of amino acids from the cells when they are incubated at lowered pH.

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REFERENCES AND NOTES

1. Bradley, M. O., Hayflick, L., and Schimke, R. T.: Protein degradation in human fibroblasts (WI-38): Effects of aging, viral transformation and amino acid analysis. J. Biol. Chem., *251:* 3521 (1970).
2. Coon, H. G., and Weiss, M. C.: A quantitative comparison of formation of spontaneous and virus produced hybrids. Proc. Natl. Acad. Sci. U.S.A., *62:* 852 (1969).
3. DeDuve, C., DeBarsy, T., Poole, B., Trouet, A., Tulkens, P., and VanHoof, F.: Lysosomatropic agents. Biochem. Pharmacol., *23:* 2495 (1974).
4. Lie, S. O., McKusick, V. A., and Neufeld, E. A.: Stimulation of genetic mucopolysaccharidoses in normal human fibroblasts by alteration of pH of the medium. Proc. Nat. Acad. Sci. U.S.A., *69:* 2361 (1972).
5. Lie, S. O., and Schofield, B. H.: Inactivation of lysosomal function in normal cultured human fibroblasts by chloroquine. Biochem. Pharmacol., *22:* 3109 (1973).
6. Lie, S. O., Schofield, B. H., Taylor, H. A., and Doty, S. B.: Structure and function of the lysosomes of human fibroblasts in culture: Dependence on medium pH. Pediatr. Res., *7:* 13 (1973).
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the folin phenol reagent. J. Biol. Chem., *193:* 265 (1951).
8. Oshima, R. G., Rhead, W. J., Thoene, J. G., and Schneider, J. A.: Cystine metabolism in human fibroblasts: comparison of normal, cystinotic and suspected γ-glutamyl cysteine synthetase deficient cells. J. Biol. Chem., *251:* 4287 (1976).
9. Reijngoud, D-J., and Tager, J. M.: Chloroquine accumulation in isolated rat liver lysosomes. FEBS Lett, *64:* 231 (1976).
10. Rubin, H.: pH and population density in the regulation of animal cell multiplication. J. Cell. Biol., *51:* 686 (1971).
11. Schneider, J. A., Schulman, J. D., Seegmiller, J. E.: Cystinosis and the Fanconi Syndrome. In: J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson: The Metabolic Basis of Inherited Disease, 4th ed. p. 1660 (McGraw-Hill Book Company, New York, 1978).
12. Schulman, J. D.: Cystinosis. DHEW Publication (NIH) 72-249. (Washington, D.C., Government Printing Office, 1973).
13. Thoene, J. G., Oshima, R. G., Crawhall, J. C., Olsen, D. C., and Schneider, J. A.: Intracellular cystine depletion by aminothiols *in vitro* and *in vivo*. J. Clin. Invest., *58:* 180 (1976).
14. Thoene, J. G., Oshima, R. G., Ritchie, D. G., and Schneider, J. A. Cystinotic fibroblasts accumulate cystine from intracellular protein catabolism. Proc. Nat. Acad. Sci. U.S.A., *74:* 4505 (1977).
15. Wibo, M., and Poole, B.: Protein degradation in cultured cells II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradattion and cathepsin B_1. J. Cell. Biol., *63:* 430 (1974).
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