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cysteamine leukocyte
cystinosis pantetheinase
fibroblast

Pantetheinase Activity and Cysteamine Content in Cystinotic and Normal Fibroblasts and Leukocytes

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Summary

Cysteamine is the most effective agent known for the reduction of the elevated cystine content of cells from patients with cystinosis. A defect in endogenous cysteamine generation could account for many of the metabolic features of this disorder. To test this hypothesis, we have developed improved methods for measuring pantetheinase (cysteamine-generating) activity and intracellular cysteamine levels and used these methods to measure such parameters in cystinotic and normal leukocytes and cultured skin fibroblasts. Pantetheinase activity as defined in the text was similar in extracts of cystinotic and normal cells [leucocytes, normal, 78 ± 15 (S.E.), cystinotic, 56 ± 6.4; fibroblasts, normal, 9.4 ± 1.5; cystinotic, 7.7 ± 1.7]. Cysteamine levels were normal in leukocytes from cystinotics receiving no cysteamine or doses of oral cysteamine too low to reduce leukocyte cystine content. The results indicate that the cause of cystinosis is unlikely to be related to a failure to generate or sustain normal intracellular cysteamine levels.

Speculation

Cysteamine is an extremely effective cystine depleting agent for cystinotic fibroblasts *in vitro* and can greatly reduce cystinotic leukocyte cystine content *in vivo.* Its pharmacologic properties suggest that it might prove to be of value in the therapy of cystinosis. However, we do not believe that a defect in endogenous cysteamine generation is a characteristic of cystinotic cells. The eventual elucidation of the cystinotic defect may require analysis of the permeability characteristics of cystinotic lysosomes or the discovery of presently unidentified pathways for lysosomal metabolism of cystine.

Cystinosis, a metabolic disease inherited as an autosomal recessive trait, is characterized by abnormal intralysosomal accumulation of L-cystine in most cells of the body (32, 36). The progressive accumulation of cystine leads to eventual destruction of cells, most notably in the kidney, and terminal renal failure by the second decade of life is characteristic of the most severe and common nephropathic variant. The fundamental biochemical defect underlying this disorder has yet to be elucidated.

The apparently major mechanism for reduction of cystine in normal cells involves enzymatically catalyzed glutathione-cystine transhydrogenation. Studies of this and other disulfide reducing systems have failed to define the abnormality causing cystinosis (28, 29, 44–47).

Cysteamine [mercaptoethylamine (MEA)] is a naturally occurring, freely soluble sulfhydryl compound which readily penetrates many biologic membranes. When supplied exogenously, it is highly effective in reducing the elevated cystine content of cystinotic cells *in vitro*, presumably by entering the cystinotic lysosomes and reducing cystine to the smaller and more soluble cysteine. A suggested mechanism for cysteamine-mediated cystine depletion has been discussed previously (42). In this model, cysteamine acts as a hydrogen carrier between cytoplasmic-reduced glutathione (which appears to penetrate membranes poorly) and intralysosomal cystine. A genetic deficiency in endogenous cysteamine generation might therefore lead to diminished capacity for intralysosomal reduction of cystine to cysteine, a molecule to which the lysosomal membrane should be permeable (33) and hence could be the fundamental etiology of abnormal cystine accumulation in cystinosis.

Cysteamine (Fig. 1) is generated in animals by only one known mechanism: the irreversible enzymatic cleavage of pantetheine (PTSH). Pantetheinase activity has been demonstrated by Abiko and associates (1–3, 26) by Dupré *et al.*, and by Cavallini in studies on a limited variety of mammals (4, 6, 8, 9, 11, 14–17). There is some evidence that pantetheinase is present in both soluble and lysosomal-microsomal cell fractions. This enzyme has not previously been described in human cells or tissues.

Pantetheinase activity has been determined by several methods. One involves quantitation of pantothenic acid (PoA), an end product of PTSH cleavage, using a microbiological assay in which PoA is a necessary growth factor (*e.g.*, using *Lactobacillus arabinosus*) (2, 5, 9, 10, 39). The radiochemical assay of Dupre *et al.* (6, 14–17) utilizes labeled PTSH as substrate and measures radioactive product after chromatographic separation from PTSH. A pH-Stat method has also been proposed (11). The first method is time consuming and proved erratic in our hands, whereas the radiochemical assay requires an expensive radiolabeled substrate which must be specially synthesized. We therefore developed a new assay system for determination of low levels of pantetheinase activity in small cell samples; our assay involves rapid quantitation of MEA on an amino acid analyzer after generation from PTSH and reaction with *N*-ethylmaleimide (NEM).

The present report describes our use of this new method to determine panthetheinase activity and define certain properties of this enzyme in extracts of cultured skin fibroblasts and freshly harvested leukocytes from normal and cystinotic subjects. We also attempted to directly measure cysteamine levels in these cells.

MATERIALS AND METHODS

TISSUE CULTURE

Skin fibroblasts matched for passage number from normal, cystinotic, and heterozygous (for cystinosis) subjects were grown to confluency at 37°C in a 5% CO_2/95% air atmosphere in roller bottles or 75 cm^2 plastic Falcon tissue culture flasks. They were cultured in Eagle's minimal essential medium with added nonessential amino acids, 2 mM glutamine, 10% fetal calf serum, and 100 μg/ml neomycin, penicillin, and streptomycin. At harvesting, the medium was decanted, the monolayer was washed three times with isotonic phosphate-buffered saline, and the cells were detached with brief exposure to 0.25% trypsin in PBS at 37°C. A small amount of fresh medium was added to stop the reaction, and the detached cells were collected and washed three times with cold phosphate-buffered saline using repetitive low-speed centrif-

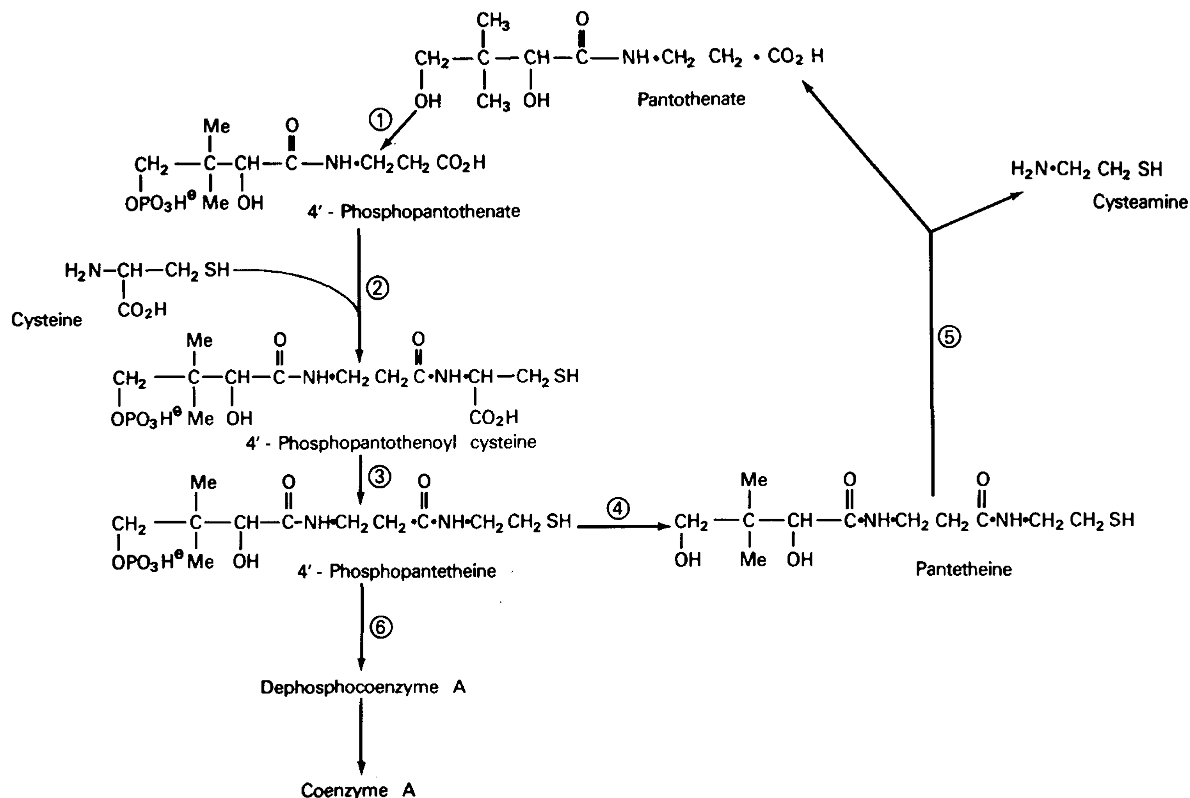

Fig. 1. The pantetheinase reaction (reaction 5) and related metabolic interconversions.

ugation and gentle resuspension. The pellet was then sonicated in assay buffer at 4°C in 10-sec bursts for a total of 60 sec. The sonicate was kept at 4°C and used within several hours for enzyme assay. Total protein was determined by the method of Lowry *et al.* (23).

LEUKOCYTE [WHITE BLOOD CELL (WBC)] PREPARATION

Five to 10 ml of freshly drawn heparinized blood was obtained from normal volunteers, cystinotic patients, and their parents (obligate heterozygotes). Blood was mixed with an equal volume of 4% dextran or Plasmagel. After sedimentation at 25°C for approximately 30 min, the supernatant was collected, the WBC concentrated by centrifugation, and the residual erythrocytes removed using three hypotonic lyses (30, 37). The WBC pellet was then resuspended in cold buffer and sonicated and used like the fibroblasts.

ASSAY TECHNIQUES

All reagents were highest purity or Analar grades. Commercially obtained pantethine (PTSS) was checked for purity using a Beckman 121M amino acid analyzer and high-voltage electrophoresis and found to have a variety of contaminants. Repurification was achieved using Dowex 50W, 8X 200 to 400 mesh, H+ resin (Biorad) in a 0.6 x 4 cm column. One ml of a 50 mg/5 ml H_2O stock solution of PTSS (kept frozen) was applied to the column and eluted with 1 ml of 0.1 N HCl and 2.5 ml of H_2O. Buffer was added to give a final PTSS concentration of 4 mM, and pH was adjusted to 7.6 for use in the assay. [Except where noted, buffer was 0.05 M potassium phosphate (pH 7.6)].

To reduce PTSS to PTSH, dithiothreitol (DTT) was used. Like PTSS, the DTT was passed over a Dowex 50 column and eluted with water. It was then diluted in buffer to give an 8 mM solution and was used immediately in the assay. Reducing power is lowered approximately 10% (as checked by DTNB) by this purification.

In the pantetheinase assay, all solutions were equilibrated with nitrogen, and the reaction was carried out under nitrogen in a 1.5 ml capped Beckman conical Microfuge tube. PTSS (0.2 μmoles) and 0.4 μmoles DTT in buffer were combined and vortexed, and after 5 min EDTA was added to a final concentration of 10^{-3} M. EDTA was used to inhibit possible subsequent oxidation of generated cysteamine to hypotaurine (7, 12, 13–20, 22, 48, 50). After 10 min of preincubation at 37°C, cell extract in buffer preequalibrated with nitrogen was added, the tube was gassed with nitrogen, and the cap was tightened. Final rection volume was 0.5 ml. The mixture was generally incubated for 90 min (WBC) or 240 min (fibroblasts) with oscillation in a water bath at 37°C; inversion of the tube was carried out every 30 min. At the conclusion of incubation, the reaction mixture was placed on ice and an additional 0.2 μmole of buffered DTT (8 mM) was added. After 10 min, 2 μmoles NEM (20 mM in buffer) were added with mixing. After 15 min, the reaction was stopped with ¼ volume of 20% sulfosalicylic acid. After centrifuging, the reaction tube in a Beckman Microfuge for 5 min, NEM-MEA was quantitated in the supernatant by automated ion exchange chromatography using a Beckman 121 M amino analyzer.

The conditions below were used on the amino acid analyzer to permit quantitation of NEM-MEA, and by utilizing two columns and automatic sample injection, it was possible to assay an average of one sample every 35 min. Jacketed 15 cm column assemblies were packed with Durrum DC-6A resin to form a 0.28 by 7.0 cm bed. Column temperature was 50°C. One hundred μliters of sample were injected onto the column. Flow rate was adjusted to approximately 10.5 ml/hr. All buffers contained 5 drops of pentachlorophenol solution, 5 mg/ml in ethanol (Pierce Chemical Co.), and 0.6 to 1.2 ml of Brij 35 detergent, 30% solution (Pierce Chemical Co.) per liter. The sequence and timing of sodium citrate buffers were as follows: 0.44 N (pH 4.00) for 3 min; 0.40 N (pH 5.03) for 7 min; and 1.0 N (pH 6.40) for 40 min. NEM-MEA eluted as an isolated peak with a retention time of approximately 47 min. NEM-MEA (0.1 to 0.2 nmoles) applied to the column was the minimum which could be reliably quantitated. NEM-MEA standards were made using known amounts of MEA, adding DTT, and reacting with excess NEM under nitrogen.

Appropriate blanks for the panthetheinase assay contained either no enzyme or enzyme which had been boiled for 60 sec; both gave equivalent results and yielded no measurable NEM-MEA. Appropriate internal and external standards of NEM-MEA

and MEA were used to confirm the quantitative nature and reliability of the assay. Addition of cystine to levels expected in cystinotic cell extracts did not affect activity in this assay.

ATTEMPTS TO ASSAY MEA BY HIGH-VOLTAGE ELECTROPHORESIS (HVE)

In preliminary experiments, NEM-MEA as well as other sulfhydryl, disulfide, mixed disulfide, and NEM-sulfhydryl amino acid derivatives used as standards were spotted on Whatman 3 MM paper, subjected to electrophoresis for 2 hr at 4000 volts using a 7.8% formic acid bath, and identified after ninhydrin staining. Rough quantitation of MEA could be achieved in this system if the MEA was previously reacted with radiolabeled NEM (either ^{14}C or ^{3}H) of known specific activity (24), an aliquot spotted on top of a nonradioactive NEM-MEA standard, and after HVE, the area corresponding to NEM-MEA dried, cut out, and radioactivity measured in a scintillation counter. To confirm identification, the radioactive NEM-MEA spot could be eluted, reapplied to Whatman 3 MM, followed by ascending chromatography in butanol/acetic acid/H_2O (12/3/5). However, attempts to accurately quantify the MEA produced in the pantetheinase assay using radioactive NEM followed by HVE yielded results much inferior to the amino acid analyzer measurement of unlabeled end product described above.

ASSAY OF CYSTEAMINE LEVELS IN CYSTINOTIC AND NORMAL CELLS

Leukocytes, usually from 20 ml heparinized blood, were prepared by dextran or Plasmagel sedimentation and hypotonic lysis as described above. The leukocyte pellet in 250 λ of 0.9% sodium chloride was sonicated for 5 to 10 sec and mixed with 50 λ of DTT solution (23.1 mg/ml) in 100 mM potassium phosphate buffer (pH 8.0). After 10 min, 150 λ of freshly dissolved NEM solution (24 mg/ml) in 10 mM potassium phosphate (pH 8.0) was added. Fifteen to 20 min later, the sample was acidified with 50 λ of 40% SSA in water. After centrifugation, NEM-MEA was determined on the amino acid analyzer as described above. Fibroblast cysteamine levels were examined analogously on washed, trypsinized cells. The method gives reliable quantitation of an internal MEA standard from a fraction of a nanomole to at least 200 nm/ml.

RESULTS

PANTETHEINASE ACTIVITY

NEM-MEA generation was assayed varying the duration of incubation, amount of cell extract, pH, and substrate concentrations to define enzyme characteristics and optimal assay conditions and to explore the possibility of differences in pantetheinase activity between normal and cystinotic cells. Because of the limited availability of fresh cystinotic WBC not all of the above variables were examined using such cells, although all were investigated using normal WBC and normal and cystinotic fibroblast sonicates.

Figure 2 shows the time course for generation of MEA from PTSH for pooled normal and cystinotic fibroblast sonicates at pH 7.6. Near linearity is maintained to at least 240 min in fibroblasts and 90 min in WBC and easily measurable amounts of MEA are produced at these times. In subsequent assays, these times of incubation were selected unless otherwise specified. There was no difference between the time course of normal and cystinotic fibroblast extracts. Within these time periods, there was strict proportionality of MEA production to amount of cell extract per assay up to at least 1 mg cell protein from normal and cystinotic fibroblasts and at least 2 mg protein from normal leukocytes. Subsequent assays were performed using 0.5 to 1 mg cell protein per assay. Replicate assays varied by less than 5%.

As summarized in Table 1, pantetheinase activity under these conditions was lower in fibroblasts than in WBC, but no substantial differences between cystinotic and normal cell extracts were noted.

Figure 3 shows the patterns of pantetheinase activity in cystinotic and normal fibroblast extracts over a wide pH range. The pH optimum is broad and similar in normal and mutant cell sonicates and in fibroblasts and WBC. Relationships between enzyme activity and substrate concentration were quantitated at pH 6.5 for fibroblasts and at pH 7.0 for WBC. There were no significant differences between the normal and cystinotic samples in apparent computer-derived K_m for substrate in either fibroblasts or WBC (Table 1).

All the data points used in constructing the table and figures represent the mean of at least duplicate, and usually triplicate, determinations.

Because some of the cystinotic children were receiving ascorbic acid during this study and some were not (31), ascorbic acid in amounts both appropriate to and in excess of the estimated blood level were added to both normal and cystinotic WBC and fibroblast extracts in certain control experiments. These amounts of ascorbate did not affect pantetheinase activity (data not shown).

INTRACELLULAR CYSTEAMINE LEVELS

Endogenous cysteamine was always less than approximately 0.2 nmole/mg cell protein in cystinotic and normal fibroblasts. It was thus not possible to test directly whether these cystinotic cells were characterized by cysteamine deficiency. However, when leukocytes were obtained from cystinotic patients who were receiving low doses of oral cysteamine (42, 43) and in whom leukocyte cystine content was still grossly elevated, it was possible to quantitate intracellular cysteamine levels. Such values could also be measured in untreated normal and cystinotic WBC's. These data are summarized in Table 2. These results suggest that intracellular cysteamine deficiency is not the cause of cystinosis. The levels of cysteamine in washed cell pellets from untreated cystinotics and from patients receiving exogenous cysteamine in low doses were generally at least as high as those found in cells from normals, yet

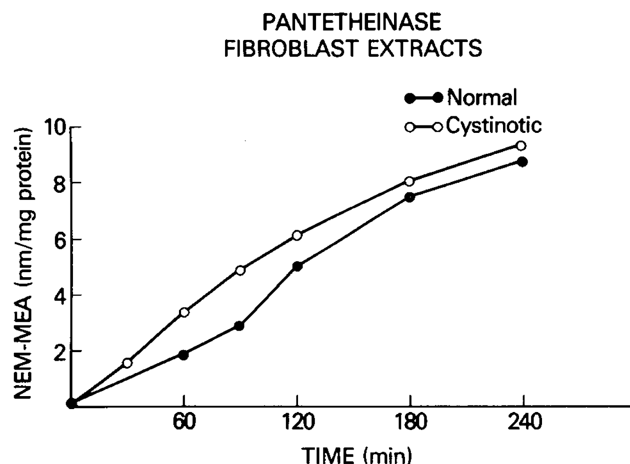

Fig. 2. The time course for generation of cysteamine in normal and cystinotic fibroblast extracts. See text for details.

Table 1. *Pantetheinase characteristics in normal and cystinotic cells*

Type cell	Cysteamine-NEM	Km[mM]	pH optimum
WBC			
Normal	78 ± 15[1] (5)[2]	0.44 ± 0.09 (5)	6.5–7.5
Cystinotic	56 ± 6.4 (4)[2]	0.28 ± 0.07 (4)	6.5–7.5
Fibroblasts			
Normal	9.4 ± 1.5 (6)[3]	0.11 ± 0.04 (4)	6–7
Cystinotic	7.7 ± 1.7 (6)[3]	0.03 ± 0.01 (3)	6–7

[1] Mean ± S.E.
[2] nmole/mg protein/90 min.
[3] nmole/mg protein/240 min. (no. of lines). All differences, $P > 0.05$.

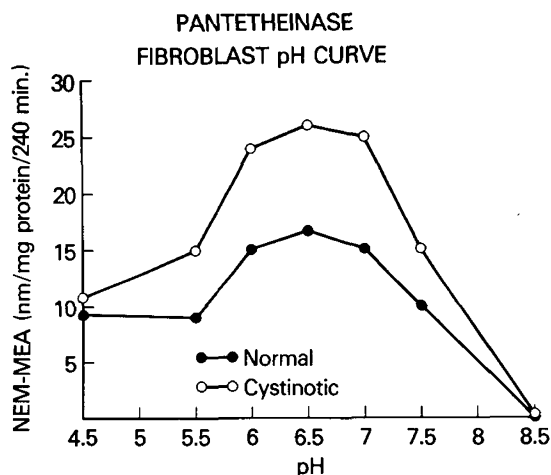

Fig. 3. Pantetheinase activity as a function of pH in cystinotic and normal fibroblast extracts.

Table 2. *Cysteamine and cystine content of leukocytes from normal and cystinotic subjects and cystinotics receiving oral cysteamine at doses which did not reduce leukocyte cystine content*

	No. of subjects	Cysteamine (range) (nmole/mg protein)	Cystine (range) (½ nmole/mg protein)
Normal	5	0.27–1.1	<0.5
Cystinotic, no cysteamine therapy	5	<0.2–4.5	2.0–12.4
Cystinotic, low dose cysteamine (10 mg/kg/day)	4	1.5–3.5	3.1–21

the former simultaneously had grossly elevated leukocyte cystine levels whereas the latter did not.

DISCUSSION

There is no evidence for decreased enzymatically catalyzed cystine reduction or metabolism in cystinotic cells (21, 27–29, 41) and little support for the presence of significant amounts of cystine-reducing activity in either normal or cystinotic lysosomes (35, 40, 46, 47, 49). Cystinotic cells have normal reduced glutathione content (27, 34). Because cysteamine supplied exogenously can reduce cystinotic intracellular cystine levels to near normal (42), the possibility that cystinosis might be due to a defect in endogenous MEA generation needed critical evaluation. Demonstration of a defect in MEA production in cystinosis might be accomplished by measurement of pantetheinase activity and/or of cell MEA levels.

The only known source of endogenous MEA is breakdown of pantetheine, involved in coenzyme A catabolism (Figure 1). This degradative pathway permits cysteamine generation and pantothenate recovery. Enzymatic degradation of CoA was described by Lipmann *et al.* and Novelli *et al.* (25) and subsequent important studies related to pantetheine catabolism have been performed by Dupre *et al.* (using horse kidney) (4, 6, 8, 11, 13–17) and Abiko *et al.* (rat liver and kidney) (1–3, 26, 36). In rat liver, CoA is degraded to phosphopantetheine by a lysosomal acid phosphatase and a nuclear and microsomal plasma membrane pyrophosphatase. Further dephosphorylation to PTSH probably occurs before a specific and irreversible amidase (pantetheinase) catalyzes cleavage to PoA and MEA. Pantetheinase activity is located primarily in the microsomal-lysosomal fraction of rat liver and rat kidney (2).

Measurement of MEA levels directly in rodent tissues has been reported by Huxtable *et al.* (20). MEA reacted with phthalic anhydride-1-^{14}C to yield N-(2-thioethyl)-phthalimide-^{14}C, which was isolated by thin-layer chromatography and identified by mass spectrometry. Estimated levels of MEA in rats were 0.17 nmole/g tissue in kidney, 0.21 in liver, 0.25 in muscle, 0.68 in brain and 2.61 in heart. With this method, large tissue samples were required, and recoveries of exogenous MEA were less than 3%. These studies, however, provided evidence that MEA is an endogenous metabolite.

We describe here a new method for measuring cysteamine either directly in biologic samples or as an end product of pantetheinase activity using reduction with DTT, reaction with NEM, and quantitation of MEA-NEM on an amino acid analyzer. The method is rapid, nonradioactive, and easy to use on small samples. Exogenous MEA is quantitatively recovered in this system.

Utilizing this assay system, cystinotic cell extracts were not different from normal with regard to total activity, apparent K_m, or pH optimum of pantetheinase. These observations suggest a normal capacity for cysteamine generation in cystinotic cells and are the first of which are aware to demonstrate PTSH cleavage activity in human tissues.

Furthermore, we were able to directly measure MEA content in leukocytes from normal subjects and from cystinotic patients receiving either no cysteamine or doses of cysteamine inadequate to normalize their leukocyte cystine content. These results also provide no support for the hypothesis that cystinosis is caused by endogenous cysteamine deficiency.

The studies of human pantetheinase activity reported here are derived from analysis of unpurified preparations. Further characterization of pantetheinase purified from human tissues would be of considerable interest, but is unlikely to alter the fundamental conclusion of our studies with regard to cystinosis. The present investigations indicate that a defect in cysteamine generation is most unlikely to represent the fundamental molecular lesion in this disease.

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51. Presented in part at the annual meeting of the American Society of Human Genetics, Vancouver, B.C., October, 1978.
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