INCREASED FREE-CYSTINE CONTENT OF FIBROBLASTS CULTURED FROM PATIENTS WITH CYSTINOSIS

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The biochemical abnormality leading to the accumulation of cystine crystals in the bone marrow, cornea and internal organs of children with the rare hereditary disease, cystinosis, is not known (Bickel et al., 1952; Patrick, 1962). The only evidence of an abnormality of cystine metabolism in this disease has been the presence of cystine crystals. Recently, however, the free-cystine content of leukocyte preparations from these children has been found to be 80 times greater than normal, even though the white cells assayed were free of crystals (Schneider, Bradley and Seegmiller, 1967). Leukocytes from parents contained six times the normal cystine content, providing the first biochemical identification of the heterozygote. Following subcellular fractionation of cystinotic leukocytes, three-quarters of the intracellular cystine was found in the granular (acid phosphatase-rich) fraction. We have now found that skin fibroblasts maintained in culture from individuals who are homozygous and heterozygous for cystinosis can also be distinguished from normal on the basis of their free-cystine content. As in cystinotic leukocytes, most of the intracellular cystine of cystinotic fibroblasts was found in the "granular" fraction of the cell.

METHODS. Primary fibroblast cultures were established from a 3 mm skin biopsy and were grown in minimum essential medium (Eagle, 1959) containing 10% heat-inactivated fetal calf serum, non-essential amino acids and neomycin sulfate (50μg/ml). In some studies, cystine was entirely omitted from the growth media and dialyzed fetal calf serum was used. Cells were grown as monolayers on glass in 32-oz. prescription bottles, in 5% carbon dioxide in air. In preparation for amino acid analysis, the cells were washed four times with 5 ml normal saline at 4°C while they were still attached to the bottle, removed from the glass by treatment for three minutes at 37°C with 5 ml 0.25% trypsin in a balanced salt solution and

washed three more times with 10 ml normal saline at 4°C. They were centrifuged between washes at 120 g for 15 seconds in conical centrifuge tubes. The cells were then lysed with a Branson sonifier Model LS75, at 2 amperes for 15 seconds, the protein immediately precipitated with 3% sulfosalicylic acid and the cystine assayed with a Beckman Model 120B amino acid analyzer, as previously described (Schneider, Bradley, and Seegmiller, 1967). Acid phosphatase and protein were assayed by the methods of Lowry (Lowry, 1957; Lowry et al., 1951).

RESULTS AND DISCUSSION. Cystinotic fibroblasts could not be distinguished from normal by their growth characteristics or appearance under phase microscopy, but the free-cystine content of these fibroblasts was over 100 times normal as shown in Table I.

TABLE I

Free-Cystine Content of Skin Fibroblasts
μmole 1/2 cystine per gram protein

Control Subjects	Age of Cells[a]	Cystine		Heterozygotes	Age of Cells	Cystine	
W.J.	V-3	<0.02[b]	<0.09	W.R.	III-4	0.77	0.72
"	VI-7	0.14		"	VI-4	0.85	
"	VI-12	0.10		"	VII-4	0.54	
G.J.	VII-7	0.15		R.M.	IX-4	0.12	
D.R.	III-5	<0.01		M.P.	II-4	0.25	
W.P.	VII-4	<0.01	<0.01	S.M.	II-4	0.26	
"	VII-8	<0.02			mean -	0.34[c]	
M.O.	VII-7	<0.03					
T.B.	XIII-7	0.11		Cystinotics			
B.P.	XIII-7	<0.02		S.R.	V-8[d]	4.87	6.64
H.	VI-6	0.13		"	VII-3	7.35	
S.	X-4	0.07		"	IX-4	7.69	
	mean -	<0.07[c]		T.M.	III-4	8.21	
				W.R.	IV-4	10.6	
				I.M.	IV-4	8.50	
				C.M.	IV-4	8.84	
				M.P.	IV-4	7.60	
					mean -	8.40[c]	

[a] Roman numeral indicates passage number; arabic numeral indicates the number of days since the last passage.

[b] In many of the control cells the cystine peak was too small to calculate; in these cases the highest cystine content possible was determined, based on the known sensitivity of the assay and the protein content of the sample being measured.

[c] Mean of individual subjects, not of total measurements.

[d] By 8 days many cells had separated from the glass, apparently no longer viable; this may have caused the lower cystine content seen here.

The value in heterozygous cells was about 5 times normal, a significant difference by the Student "t" test ($p \sim 0.01$). The mean free-cystine content of the three groups was remarkably similar to that found in leukocytes, as summarized in Table II.

TABLE II

Comparison of Free-Cystine Content of Skin Fibroblasts and Leukocytes
μmoles 1/2 cystine per gram protein

	Skin-fibroblasts		Leukocytes	
Cystinotics	8.40 ± 1.33*	(6)**	6.44 ± 2.76	(9)
Heterozygotes	0.34 ± 0.26	(4)	0.49 ± 0.28	(9)
Control subjects	0.07	(9)	0.08 ± 0.06	(10)

*Mean ± 1 S.D. **Number of subjects studied.

As in leukocytes, most of the intracellular free-cystine was found in the granular fraction after subcellular fractionation. A typical experiment is shown in Table III.

TABLE III

Sub-cellular Fractionation of Cystinotic Fibroblasts

Fraction	Acid-Phosphatase μmole pNP/30'/mg protein	Percent total	Cystine μmole 1/2 cystine/ gm protein	Percent total
Granular	3.3	50.	81.	83.
Supernatant	0.47	50.	2.3	17.

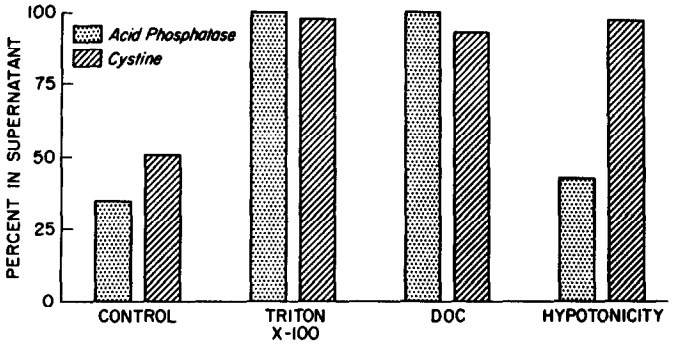
Skin fibroblasts were from S.R. (X-4). Following lysis by sonication in 4 ml of 0.25 M sucrose as described in "Methods", the granular fraction was separated by centrifugation at 27,000 g for 10 minutes. The granular pellet was taken up in 4 ml of 0.25 M sucrose and an aliquot from each fraction was treated with Triton X-100 (Rohm and Hass, Philadelphia, Pa.) prior to acid phosphatase assay (see Methods). The remainder of each fraction was prepared for amino acid analysis as described in "Methods".

In a separate experiment, cystinotic fibroblasts were lysed, divided into four equal parts and each treated differently before fractionation, as shown in Figure 1. Treatment with detergent released both cystine and the lysosomal enzyme acid phosphatase from the 'granules'. By contrast, treatment of another portion with hypotonic sucrose released substantially

more cystine than acid phosphatase. These results suggest, but do not prove, that compartmentalization is in a subcellular component other than lysosomes. In addition, this experiment argues against the location of cystine in peroxisomes, a different subcellular component (de Duve and Baudhuin, 1966) which, in rat liver preparations, is more resistant than lysosomes to hypotonicity (Baudhuin et al., 1965).

FIGURE 1

Release of Cystine and Acid Phosphatase
from the Granular Fraction of Cystinotic Fibroblasts



Skin fibroblasts from W.R. (XII-4) were lysed as in "Methods" in 4 ml of 0.25 M sucrose and then divided into four equal parts. One portion served as control; one was made to 0.01% with Triton X-100; one was made to 1.0% with deoxycholate (DOC) and the last part was treated with 5 times its volume of distilled water. After 15 minutes at 4°C, the granular fractions were separated, and acid phosphatase and cystine assayed as described in Table III. The total recovery of each was nearly identical in the 4 portions.

Cystine is an essential amino acid for cells in culture (Eagle et al., 1960). If the cystine contained in cystinotic fibroblasts were available to the cells, they might grow better than normal cells in a cystine-free media. After 3 days growth, cystinotic and normal fibroblasts were washed three times with cystine-free media (see Methods) and then allowed to grow in this media. Within 12 hours, both the cystinotic and normal cells came loose from the glass, apparently no longer viable; although if cystine were added to the same media at the time of transfer, normal growth occurred. The cystinotic cells which had detached from the bottle still contained essentially the same increased amount of cystine as had been previously measured in this cell line. Thus, the cystine in these cells is compart-

mentalized in such a way as to not be available to the cell, even when needed

SUMMARY. The presence of a significantly increased content of free-cystine in skin fibroblasts from both homozygotes and heterozygotes for cystinosis emphasizes the central role of cystine in this disease, even though the primary defect responsible for cystine accumulation is yet to be determined. The studies described in this communication provide evidence that cystine is compartmentalized in a subcellular location in cystinotic cells. In fact, the very growth of cystinotic fibroblasts in the presence of more than 100 times the usual content of free-cystine is evidence that the accumulated cystine is not freely dispersed throughout the cell, since it would otherwise inhibit many enzymes requiring free sulfhydryl groups for activity (Patrick, 1965). We have no evidence as to whether the cystine is located in a known subcellular organelle or in a previously unrecognized location. Skin fibroblasts may provide a convenient tool to pursue these questions.

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