## Increased Cystine in Leukocytes from Individuals Homozygous and Heterozygous for Cystinosis

Abstract. *In patients with cystinosis, the concentration of free cystine in leukocytes was 80 times greater than normal, and six times the normal content for their parents. This is the first demonstration of an abnormality in heterozygotes for this rare inherited disease of childhood. Three-quarters of the cystine was recovered in the granular fraction of cystinotic leukocytes.*

The chemical abnormality leading to the accumulation of cystine crystals in bone marrow, cornea, and internal organs in children with the rare hereditary disease cystinosis is not known (*1*). It is thought that cystine causes both the renal tubular defects of the Fanconi syndrome, which is the first symptom of this disease, and the progressive renal glomerular damage leading to their death in late childhood (*2*). While an extracellular origin for the crystals is suggested by their occurrence in organs rich in reticuloendothelial cells where they might be trapped by phagocytosis, the fact that the cystine concentration in the plasma of these children is within the normal range and far below the saturation concentration disputes this possibility (*3*).

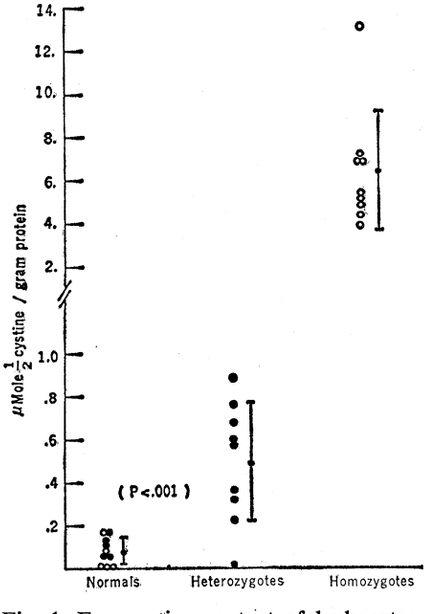


Fig. 1. Free-cystine content of leukocytes. From 5 to 40 ml of blood was mixed with 20 times its volume of normal saline (4°C) to prevent clotting (*13*). The cells were separated by centrifugation at 700*g* for 20 minutes and then were given two hypotonic shocks and one wash in normal saline. Care was taken to remove erythrocyte ghosts which were found on the surface of the final leukocyte pellet. The leukocytes were lysed by sonication, and the protein was precipitated with 3 percent sulfosalicylic acid. The cystine was assayed with a Beckman model 120B amino acid analyzer by means of a gradient elution technique (*8*). Protein was determined by the method of Lowry (*14*). Children are represented by open circles, and adults are represented by filled circles.

An intracellular origin for the crystals was suggested by Barr after careful histological study of postmortem tissue (*4*), and a high content of cystine has been reported in such tissue (*3, 5*). These high values, however, undoubtedly reflect the presence of crystalline cystine deposits. Evidence of a high intracellular cystine concentration, required for crystal formation, has not been previously presented.

The free cystine content of peripheral leukocytes was substantially higher than normal in patients with cystinosis and moderately increased in their parents (Fig. 1). The cystine content of leukocytes from nine infants and children with cystinosis was 6.44 ± 2.76 μmole of 1/2 cystine per gram of protein (mean ± 1 S.D.) as compared to the normal value of 0.081 ± 0.064 (*6*). The cystine content of leukocytes in nine obligate heterozygotes (parents) was 0.490 ± 0.280. Only one heterozygote showed a value in the normal range (Fig. 1). The difference between the normal and heterozygous values was highly significant ($P < .001$).

Occasional cystine crystals are seen in leukocytes from simple buffy-coat preparations from blood of patients with cystinosis (*7*), but careful search by phase microscopy of the leukocyte preparations used in these measurements revealed no crystals. The fact that leukocytes from heterozygotes, in whom cystine crystals have never been seen, contain increased amounts of this amino acid is additional evidence for a primary derangement of metabolism rather than phagocytosis of crystals.

In most studies, a sample of the leukocyte preparation was lysed in the presence of an excess of *N*-ethylmaleimide (NEM) so that any of the reduced form, cysteine, could be measured separately from cystine. Although we used a system of analysis that could detect as little as 1 mμmole of the cysteine-NEM derivative (*8*), none was identified in 100 mg (wet weight) of normal or heterozygous cells or 25 mg of homozygous cells. This was unexpected because cystine transported into other mammalian tissue is found intracellularly primarily in the reduced form (*9*). One explanation for this could be that cysteine is leached out of the cells during their preparation. However, these cells were prepared at 4°C, a temperature at which there is very little efflux of cysteine from leukocytes (*10*).

Table 1. Subcellular fractionation of cystinotic leukocytes. Approximately 100 mg (wet weight) of leukocytes was lysed in 4 ml of 0.25*M* sucrose with a Branson Sonifier, model LS75, 2.5 amperes for 20 seconds. The nuclear fraction was separated by centrifugation at 1000*g* for 10 minutes, and the granular fraction was separated by centrifugation at 27,000*g* for 10 minutes. No crystals were seen in the granular fraction by phase microscopy. Each fraction was brought to 4 ml with 0.25*M* sucrose, and 0.020 ml Triton X-100 (*11*) was added; 0.020 ml of each fraction was assayed for acid phosphatase (*15*), and the remainder was prepared for amino acid analysis (*8*). Acid phosphatase is expressed in micromoles of *p*-nitrophenol liberated per 30 minutes per milligram of protein; cystine is expressed in micromoles of ½ cystine per gram of protein. Numbers in parentheses represent percent of total.

| Fraction | Acid phosphatase | | Cystine | |
|---|---|---|---|---|
| Nuclear | 1.8 | (0.9) | 31.8 | (4.8) |
| Granular | 12.5 | (53.5) | 59.0 | (78.8) |
| Supernatant | 2.9 | (45.6) | 3.3 | (16.4) |

Possibly, cysteine is rapidly metabolized in vivo, but small amounts of the oxidized form, cystine, might exist in subcellular locations which are not accessible to the usual reductive mechanisms. The defect in cystinosis might then be an excessive compartmentalization of this amino acid. To test this hypothesis, cystinotic leukocytes were subjected to a simple fractionation of subcellular components (Table 1). In five fractionations of cystinotic leukocytes, 75.4 ± 6.0 percent of the intracellular cystine was found in the granular fraction, while 60 percent of the small amount of cystine in normal leukocytes was found in this fraction. In another fractionation experiment (Table 2), the supernatant and granular fractions were incubated at 37°C for 30 minutes, and then NEM was added. In the granular fraction, no cysteine-NEM was subsequently found, but in the supernatant, 80 percent of the cystine or cysteine was then found as cysteine-NEM (*8*). Thus, cystine was reduced in the soluble portion of the cystinotic cell, but not in the granular fraction where most of the cystine resides.

The total recovery of cystine in the fractionation experiments was greater than in simultaneous measurements of intracellular cystine. Because Triton X-100 (*11*) had been added to each fraction to facilitate the release of acid

Table 2. Effect of incubation on the state of cystine or cysteine in fractions from cystinotic leukocytes. Approximately 50 mg (wet weight) of leukocytes were lysed (as in Table 1) in a solution which was 0.25*M* in sucrose, 0.1*M* in tris buffer, *p*H 7.4, and $10^{-4}M$ in ethylenediaminetetraacetate. The fractions were prepared as described in Table 1, and samples were assayed for acid phosphatase. The remainder of the granular and supernatant fractions, to which no detergent had been added, were incubated in 4 ml of the buffer at 37°C for 30 minutes, and then an excess of NEM was added. The samples were then prepared for the amino acid analyzer (*8*). The smallest amount that could have been detected in the granular fraction is 0.1 μmole of cysteine per gram of protein. Acid phosphatase is expressed in micromoles of *p*-nitrophenol liberated per 30 minutes per milligram of protein; cystine is expressed in micromoles of ½ cystine per gram of protein; cysteine-NEM is expressed in micromoles per gram of protein. Numbers in parentheses represent percent of total.

| Fraction | Acid phosphatase | Cystine | Cysteine-NEM | Total of cystine and cysteine-NEM (%) |
|---|---|---|---|---|
| Nuclear | 1.7 (4.4) | 14.0 | | 12.2 |
| Granular | 8.5 (70.8) | 22.3 | < 0.1 | 62.7 |
| Supernatant | 1.0 (24.8) | 0.53 | 2.59 | 25.1 |

phosphatase from lysosomes, we performed an experiment in which this compound was added to only a portion of each fraction. In the presence of this detergent, the cystine content increased from 33.5 to 56.6 μmole of 1/2 cystine per gram of protein in the granular fraction, but only from 2.10 to 2.25 μmole of 1/2 cystine per gram of protein in the supernatant fraction. When NEM was added after Triton X-100, no cysteine-NEM was found; hence, the new cystine uncovered had been in the oxidized state.

This evidence suggests that the cystine stores in these leukocytes are separated from the soluble portion of the cytoplasm by a lipid-containing structure. This would explain why therapeutic measures which lower the cystine concentration in the plasma fail to control this disease. Treatment with D-penicillamine for 3 years or a low cystine diet for over 1 year lowered the cystine concentration in the plasma of children with cystinosis by more than 50 percent but failed to alter either the appearance of crystalline deposits in bone marrow or the clinical course of the disease (*3*). In addition, the cystine content of leukocyte was not influenced by either form of therapy (*12*).

These data suggest, but do not prove, that the primary defect in cystinosis is an abnormal subcellular compartmentalization of cystine. Another explanation of these observations could be that microcrystals of cystine form in these leukocytes and are subsequently surrounded by a lipid membrane. More exact fractionations combined with electron microscopy in leukocytes and other tissues, as well as a better understanding of the fate of cystine in normal tissues, is necessary to confirm this hypothesis. The presence of significantly higher concentrations of cystine in leukocytes from the parents of these children is compatible with a recessive mode of inheritance of this disease, as suggested by family pedigrees (*1*), and may allow the detection of the heterozygous carriers.

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14 June 1967

## Growth of a Thermophilic Bacterium on Hydrocarbons: A New Source of Single-Cell Protein

Abstract. *A species of the genus* Bacillus, *capable of growth on normal alkanes at 70°C, was isolated by the method known as enrichment culture. Preliminary analyses of its amino acid composition indicate that it would be a good source of protein for human nutrition. Possible advantages of the use of such organisms for the production of single-cell protein include simplification of fermentor cooling and asepsis.*

Recent reports (*1*) have dealt with the possibility of producing single-cell protein (SCP) by culturing yeasts or bacteria on hydrocarbon substrates. One of the technological problems to be overcome, if such production is to be economic, is the relatively large amount of heat evolved per pound of cells produced—a consequence of the large amounts of oxygen required for oxidation of hydrocarbons. It has been estimated (*2*) that, with a hydrocarbon substrate, heat production per unit mass of cells is about double what would be evolved if a carbohydrate substrate were used. Heat of fermentation is normally removed by circulating water for cooling through coils or the fermentor jacket. For this method to be effective, the water must be substantially cooler than the operating temperature of the fermentor, and this gradient must be larger when the rate of heat evolution is greater. Previous proposals for the production of SCP by growth on hydrocarbons have described growth temperatures in the range of 25° to 37°C, which would require cooling water of a temperature lower than 30°C. However, in many parts of the world for many months of the year, water temperatures exceed 30°C, and even evaporative cooling towers are ineffective because of high atmospheric humidity. Therefore, mechanical refrigeration, with consequent higher operating costs, would have to be considered.

If thermophilic organisms could be obtained, the problem of fermentor cooling would be simplified, since a large gradient between the operating temperature of the fermentor and the temperature of the cooling water would be available. Unfortunately, reports of the growth of thermophiles on hydrocarbons are scanty. Allen (*3*) failed to obtain organisms from paraffin en-