THE METABOLISM OF AMINO ACIDS, PEPTIDES, AND DISULFIDES IN LYSOSOMES OF FIBROBLASTS CULTURED FROM NORMAL INDIVIDUALS AND THOSE WITH CYSTINOSIS

By JOSEPH D. SCHULMAN, M.D. AND KATHRYN H. BRADLEY
(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 29 June 1970)

The lysosome, discovered only two decades ago (1), has been the subject of an enormous body of experimental work (2). Until recently detailed investigations of lysosomal metabolism have generally been confined to nonhuman organisms. The observations that certain heritable diseases involve the storage within lysosomes of abnormal quantities of characteristic substances have been accompanied by significant advances in the understanding of normal and abnormal lysosomal metabolism in man. A number of these storage disorders, including Pompe's disease (3), Tay-Sach's disease (4), Gaucher's disease (5), metachromatic leukodystrophy (6), GM1 gangliosidosis (7), Niemann-Pick disease (8), acid phosphatase deficiency (9), Wolman's disease (10), Fabry's disease (11), and fucosidosis (12) have been attributed to decreased activity of specific lysosomal acid hydrolases. Considerable information is now available about the enzymatic complement of human and animal lysosomes, and about the physiological importance of these enzymes (13).

Some lysosomal enzymes degrade proteins to amino acids and small peptides, but relatively little is known about the mechanism by which these small metabolites leave the lysosome. Certain cells appear capable of discharging their intralysosomal contents by exocytosis (14), but in many cells this process appears to be relatively inactive. A mechanism for penetration of the lysosomal membrane by small metabolites is presumed to be operative. Because of the fragility of isolated lysosomes, the permeability phenomena involving amino acids and peptides have been difficult to investigate directly.

In 1969, Ehrenreich and Cohn described an imaginative approach for investigating these lysosomal properties in situ in mouse peritoneal macrophages (15). If the macrophages were maintained in culture and exposed to relatively high concentrations of certain indigestible D-peptides they developed a pronounced cytoplasmic vacuolation. The mechanism of vacuolation appears to be as follows: tested compounds are ingested into the lysosomes by cellular endocytosis; if the compound cannot be metabolized within the lysosomes and cannot readily penetrate the lysosomal membrane, it accumulates within these organelles which undergo osmotic swelling and become visible as lucent vacuoles. Using this technique Ehrenreich and Cohn found that mouse
macrophage lysosomes are relatively impermeable to peptides with molecular weights of greater than 220–230.

We have found that the methods developed for the study of mouse macrophages can be adapted to permit investigation of human lysosomal metabolism in cultured skin fibroblasts. The responses of normal fibroblasts, and fibroblasts from individuals homozygous and heterozygous for cystinosis, a rare autosomal-inherited disorder of cystine metabolism, have been investigated after exposure to a variety of amino acids, peptides, and disulfide compounds.

Cystinosis is clinically manifested by the Fanconi syndrome and progressive glomerular insufficiency causing death in childhood (type 1) (16, 17), although clinical variants with absence of renal disease (type 2) (18, 19) or onset of renal manifestations after childhood (type 3) (20, footnote 1) are recognized. The tissues of cystinotic individuals contain massive amounts of cystine, which appears to accumulate intracellularly. Electron micrographs have demonstrated crystalline (21–23) or amorphous (24) inclusions within cystinotic lysosomes, and accretions of cystine in sonicates of cystinotic white cells co-equilibrate with lysosomes after isopycnic density gradient centrifugation (25). Although crystals assumed to be cystine have in a single instance been described in cystinotic mitochondria (26), the preponderance of evidence suggests that cystinosis is a lysosomal storage disease. Cystinotic fibroblasts cultured in vitro have excessive amounts of stored cystine (nearly 100 × normal) (27) and the cystine content of fibroblasts from heterozygous individuals is less markedly elevated but usually greater than normal (27); hence the characteristic metabolic lesion in cystinosis is expressed in the cultured fibroblasts, and the mechanism of cystine storage can be investigated using these cells.

In the studies reported here, the permeability characteristics of human fibroblast lysosomes have been found to be remarkably similar to those of mouse macrophage lysosomes. Cystinotic cells exhibited an abnormality in accumulation of disulfides by their lysosomes. The experiments suggest that normal lysosomes may have a mechanism for lysosomal transport or metabolism of disulfides with cysteine moieties, and that a congenital deficiency of this mechanism may be the distinctive molecular lesion in cystinosis.

Materials and Methods

Chemicals.—D-Alanine, D-phenylalanine, D-tryptophan, D-L-allocystathionine, and D-penicillamine (D-β,β-dimethylcysteine) were obtained from Mann Research Labs, Inc., New York; D-carnosine from Cyclo Chemical Corp., Los Angeles, Calif.; D-valyl-alanine, D-alanyl-threonine, D-trialanine, and L-penicillamine from Fox Chemical Co., Los Angeles, Calif.; D-cysteine and L-cysteine from Sigma Chemical Co., St. Louis, Mo.; D-penicillamine disulfide and D-cystathionine from K & K Laboratories Inc., Plainview, N. Y.

The mixed disulfides of cysteine and penicillamine were prepared by the method of Crawford (28) using Amberlite resin 1R-120H (Fisher Scientific Co., Pittsburgh, Pa.). L-Cysteine-D-penicillamine disulfide and D-cysteine-L-penicillamine disulfide were recrystallized from hot water or hot 25% ethanol, L-cysteine-L-penicillamine disulfide and D-cysteine-D-penicil-

1 Pittman, G., and J. D. Schulman. In preparation.
lamine from 50-80% ethanol. The L-L- and D-D-cysteine-penicillamine (mixed) disulfides were less stable than the L-D or D-L mixed disulfides, and seemed to undergo some oxidative breakdown during air-drying of their crystals. L-Penicillamine disulfide was prepared by a slight modification of the procedure used for the mixed disulfides, and was recrystallized from 80% ethanol.

Tissue Culture Methods.—Fibroblasts were grown in glass bottles from primary skin explants of cystinotic, heterozygous, and normal individuals by standard techniques in Eagle's medium #2 (National Institutes of Health Media Division) with 10% fetal calf serum (Industrial Biological Labs., Rockville, Md.), nonessential amino acids, glutamine, and neomycin (50 μg/ml) in an atmosphere of 95% air-5% CO2 at 37°C. Prior to exposure to a test compound the cells were detached with 0.25% trypsin in Dulbecco's phosphate buffered saline, pH 7.4 (NIH Media Division), washed once in buffered saline without trypsin, and plated at relatively low-population densities into 35 mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.) containing 12 mm glass cover slips (Arthur H. Thomas Co., Philadelphia, Pa.) and growth medium with 20% fetal calf serum.

After the cells had been in subculture for 72 hr the medium was replaced with fresh medium containing 20% fetal calf serum and one of the test compounds at the indicated concentrations (usually 0.04 μM). Media containing these compounds were adjusted to pH 7.4 with HCl or NaOH, if necessary, and sterilized by Millipore filtration (Millipore Filter Corp., Bedford, Mass.) before use. After 20-24 hr of exposure to the test compounds the cells were fixed in glutaraldehyde (15). The cover slips were gently placed into cold 1.2% glutaraldehyde (purified by exposure to activated charcoal) in Sorensen's phosphate buffer, pH 7.4 (29). After 5-7 min the cover slips were rinsed with two changes of distilled water, and the cells examined as unstained wet preparations using an inverted Nikon MS (Nikon Inc., Instrument Division, Garden City, N. Y.) phase microscope. Viewing was usually at 400 X and photographs were obtained with a Polaroid attachment.

Control cells, treated in identical fashion but not exposed to the test compound, were prepared for each cell line in each experiment and were compared with the cells exposed to that compound. This was essential to prevent false positive readings, because certain cell lines occasionally showed vacuolation in the absence of exposure to any test compound; the reasons for vacuolation under these circumstances are unclear.

Vacuolation was graded as positive or negative for the D-amino acids and peptides. To be called positive, nearly all the cells had to contain abundant and relatively large phase-lucent vacuoles after exposure to the test compound, and demonstrate negligible vacuolation in the absence of such exposure. For the tests with the disulfide compounds, the cultures are recorded in three categories: negative (unvacuolated), intermediate (up to 50% of cells with fine vacuoles), and positive (as described above) in order to classify more precisely the gradations of response to these substances.

All tests were graded by one observer (J. D. S.); cells and test compounds were coded and the examiner had no knowledge of this code until completion of his scoring of the cells. The effect of each test compound on each cell line was independently evaluated two to six times. In almost all cases these repetitive determinations were in agreement, and where this was not so is indicated in the tables.

Quantitative Disulfide Analysis.—Cystinotic and normal cells were exposed in 32 oz prescription bottles to L-cysteine-D-penicillamine disulfide under the same conditions used to test for vacuolation. The cells were then analyzed for intracellular disulfide levels by the method developed by Schneider for intracellular cystine analysis (30), using a Model 120B Beckman amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) modified for high sensitivity and employing the gradient elution system of Crawhall (31).

Purity of the various disulfides used in the vacuolation experiments was confirmed by direct quantitative analysis using the same analyzer and elution system.
RESULTS

Induction of Vacuolization in Cystinotic and Normal Cells by D-Amino Acids and Peptides.—Table I indicates the results of testing a range of amino acids and peptides on several lines of cystinotic and normal cells. D-Tryptophan and D-cystathionine were toxic to all cell lines, and vacuolation could not be graded for these compounds. D-L-Allo-cystathionine and D-carnosine were slightly toxic at the higher concentrations (0.06-0.07 M) but this degree of toxicity appeared not to interfere with observations on vacuolation. There is a striking correlation between molecular weight of the amino acids and nonbiodegradable peptides and their capacity to induce vacuolation. D-Trialanine, with a molecular weight of 230, uniformly vacuolized all cell lines. The slightly smaller D-L-Allo-cystathionine and D-carnosine molecules vacuolized both cystinotic and normal cells, but the results were somewhat variable in that certain cell lines did not show appreciable vacuolation on exposure to these compounds (Figs. 1 and 2).

The results indicate that in both cystinotic and normal cells, the critical molecular weight above which vacuolation occurs is 220-230. Cystinotic and normal lysosomes thus appear to have a similar permeability to a graded series of amino acids and peptides. Human fibroblasts behave in a remarkably similar manner to mouse peritoneal macrophages in this test system; in the mouse the critical molecular weight for vacuolation is also about 230 (15).

Induction of Vacuolation in Cystinotic, Heterozygous, and Normal Cells by Exposure to Disulfides.—The molecular weight of cystine is 240, larger than the 220-230 which induces vacuolation. L-Cystine and D-cystine are too insoluble, however, to be tested for their capacity to vacuolize. The mixed disulfides of cysteine and penicillamine (mol wt 269) are soluble enough to achieve 0.04 M

| Added compound 0.04-0.07 M | Molecular weight | Vacuolization |
|----------------------------|-----------------|---------------|
|                            |                 | Cystinotic    | Normal        |
| D-Alanine                  | 89              | 0             | 0             |
| D-Valine                   | 117             | 0             | 0             |
| D-Phenylalanine            | 165             | 0             | 0             |
| D-Val-ala                  | 188             | 0             | 0             |
| D-Ala-threo                | 192             | 0             | 0             |
| DL-Allo-cystathionine      | 222             | ±             | ±             |
| D-Carnosine                | 226             | ±             | ±             |
| D-Trialanine               | 231             | Pos.          | Pos.          |

0 = negative; ± indicates variable scores in each group; Pos. = positive.
solutions in tissue culture medium; the penicillamine disulfides (mol wt 298) are even more soluble. These compounds were therefore tested for capacity to induce vacuolation (Figs. 3-5) in normal cell lines, cell derived from children with the classical, lethal form of cystinosis (type 1), and one cell line from each

![Fig. 1. Normal fibroblasts. Phase-lucent vacuoles are not seen. Dense bodies are visible in the cytoplasm. × 700.](image)

of the rare variants of cystinosis (types 2 and 3). L-Cysteine-D-penicillamine disulfide and L-penicillamine disulfide were each tested with the largest number of cell lines, including five from individuals heterozygous for type 1 cystinosis. D-Cysteine-D-penicillamine disulfide was toxic to all cell lines, perhaps because of an unidentified impurity caused by oxidation during its recrystallization, and was not further studied. (Reduced) D- or L-penicillamine also was toxic to all cells at 0.04 mM concentrations.

L-Cysteine-D-penicillamine disulfide produced prominent vacuolation of the nine cell lines from individuals with type 1 cystinosis (Tables II and III).
Vacuolation of the cells from the two patients with the less severe variants of cystinosis was usually less striking; although most of the cells in these two lines manifested vacuolation on exposure to this compound, the size of the vacuoles was usually smaller than those observed in cells from the classical form of the disease. Cells from individuals heterozygous for type 1 cystinosis did not demonstrate positive vacuolation; however, one of these five lines exhibited intermediate vacuolation which was difficult to distinguish from the appearance of the type 3 cystinotic cells. All seven normal cell lines failed to vacuolize on exposure to this disulfide.

The same general results were obtained with the less extensively studied L-cysteine-L-penicillamine disulfide and D-cysteine-L-penicillamine disulfide. Cystinotic cells gave positive, or in certain cases mixed positive and intermediate responses, on replicate scoring. As with L-cysteine-D-penicillamine

Fig. 2. Normal fibroblasts exposed to D-trialanine. × 800.
disulfide, normal cell lines failed to vacuolize on exposure to either of these mixed disulfides.

In striking contrast to the results with the mixed disulfides, D-penicillamine disulfide or L-penicillamine disulfide produced prominent vacuolation in all tested cell lines. There was no observable tendency toward selective vacuolation in cystinotic cells with either of these test compounds.

*Intracellular Disulfide Levels after Exposure to L-Cysteine-D-Penicillamine Disulfide.*—Normal fibroblasts exposed to L-cysteine-D-penicillamine disulfide had an intracellular content of this disulfide of 3.20 μmole ½ disulfide/gram cell protein. In marked contrast, and in complete harmony with the vacuolation data previously described, the disulfide content of type 1 cystinotic cells was 67.8 μmole ½ disulfide/gram cell protein, more than twenty times greater than in the normal cells. This strongly supports the suggestion that the mixed
DISCUSSION

The present observations represent in part a systematic investigation of the capacity of normal human lysosomes to metabolize amino acids, nonbiodegradable peptides, and certain low molecular weight disulfides. Human fibroblasts in culture have been shown to be excellent subjects for such investigations. The vacuolation method developed by Ehrenreich and Cohn, when applied to the investigation of human lysosomal metabolism, indicates a striking similarity in the permeability characteristics of the lysosomes of the mouse peritoneal macrophage (15) and the human fibroblast. In both types of cells, the lysosomal membrane apparently constitutes a significant permeability barrier for amino acids and peptides with molecular weights of 220–230 or

FIG. 4. Cystinotic fibroblasts exposed to L-cysteine-D-penicillamine disulfide. X 800.
greater. These observations on different cell types from two different organisms suggest that the permeability characteristics of lysosomes in mesodermally derived cells of many mammals may be quite similar, and that the results of

![Image](image.jpg)

**Fig. 5.** Cystinotic fibroblasts treated with D-cysteine-L-penicillamine disulfide. × 800.

experimental studies of lysosomal permeability in mammals may be directly applicable to man.

These studies begin to clarify our understanding of the mechanism of cystine storage within cystinotic cells, and of the metabolism of disulfides by lysosomes. Despite evidence favoring the concept that cystine is stored within lysosomes in cystinosis (21–25), it has been difficult to understand how a metabolite as small as cystine would remain compartmentalized within the lysosomal envelope. This phenomenon can now be better understood in terms of the ex-
### TABLE II

**Disulfide-Induced Vacuolation**

| Source          | L-cyst.-D-pen. | L-cyst.-L-pen. | D-cyst.-L-pen. | D-pen. disulfide | L-pen. disulfide |
|-----------------|----------------|----------------|----------------|-------------------|------------------|
| Normal          | 0              | 0*             | 0*             | Pos.              | Pos.             |
| R. M.           | 0              | 0              | 0*             | Pos.              | Pos.             |
| R. T.           | 0              | 0              | 0              | --                | Pos.             |
| A. B.           | 0              | 0              | 0              | --                | Pos.             |
| J. W.           | 0              | 0              | 0              | --                | Pos.             |
| J. M.           | 0              | 0              | 0              | Pos.              | Pos.             |
| B. H.           | 0              | 0              | 0              | Pos.              | Pos.             |
| Heterozygotes   |                |                |                |                   |                  |
| M. B.           | Int.           |                |                |                   |                  |
| F. K.           | 0              |                |                |                   |                  |
| X. McG.         | 0              |                |                |                   |                  |
| S. M.           | 0*             |                |                |                   |                  |
| A. M.           | 0              |                |                |                   |                  |
| Cystinosis (Type 1) |                |                |                |                   |                  |
| C. K.           | Pos.           |                |                |                   |                  |
| J. G.           | Pos.           | Pos.           | Int.*          | Pos.              | Pos.             |
| M. P.           | Pos.           | Int.*          | Int.*          | Pos.              | Pos.             |
| S. McG.         | Pos.           |                |                | Pos.              | Pos.             |
| S. L.           | Pos.           | Pos.           | Pos.           | Pos.              | Pos.             |
| D. R.           | Pos.           | Pos.           | Pos.           | Pos.              | Pos.             |
| T. M.           | Pos.           |                |                | Pos.              | Pos.             |
| I. M.           | Pos.           | Pos.           | Pos.           | Pos.              | Pos.             |
| W. R.           | Pos.           |                |                | --                | Pos.             |
| Cystinosis (Type 2) |                |                |                |                   |                  |
| D. P.           | Pos.*          | Pos.           | Int.*          | Pos.              | Pos.             |
| Cystinosis (Type 3) |                |                |                |                   |                  |
| D. S.           | Int.           | Int.*          | Pos.           | Pos.              | Pos.             |

*Variable ratings, the most common or lower rating is given for each cell line. All disulfide concentrations are 0.04 M. 0 = negative; Int. = intermediate; Pos = positive.*

### TABLE III

**Composite Scores, Disulfides**

| Source          | L-cyst.-D-pen. | L-cyst.-L-pen. | D-cyst.-L-pen. | D-pen. disulfide | L-pen. disulfide |
|-----------------|----------------|----------------|----------------|-------------------|------------------|
| Normal          | 0              | 0              | 7              | 0                 | 0                |
| Heterozygotes   | 0              | 1              | 4              | --                | --               |
| Cystinosis, Type 1 | 9              | 0              | 0              | 4                 | 1                |
| Cystinosis, Type 2 | 1              | 0              | 0              | 1                 | 0                |
| Cystinosis, Type 3 | 0              | 1              | 0              | 1                 | 0                |

**Pos. = positive; Int. = intermediate; Neg. = negative.**

1099
experiments on the fibroblasts. Lysosomal membranes of the human fibroblast present a significant diffusional barrier to efflux of substances with molecular weights greater than 220–230. Cystine has a molecular weight of 240 (the highest of any naturally-occurring amino acid), and its trapping within cystinotic lysosomes appears to be explicable in terms of its molecular size. The molecule may be too large to penetrate the 4 Å pores which are thought to be present in areas of micellar organization within the lysosomal membrane (15, 29). Furthermore, cystine is insoluble in nonpolar solvents and this could be an additional factor inhibiting its diffusion through areas of the lysosomal membrane which might consist of continuous bimolecular leaflets (32); both molecular weight and lipid–water partition coefficients seem to determine the rate of penetration of permeants through artificial model membranes (33).

Normal cells, however, do not demonstrate lysosomal accumulations of cystine. It appears therefore that normal human lysosomes may contain a transport system which facilitates the efflux of cystine from the lysosomes or an enzyme which converts cystine to a smaller or more permeable molecule. In human cystinosis, one would expect that this mechanism preventing the accumulation of cystine within the lysosomes would be inoperative.

The failure of induction of vacuolation in normal fibroblasts by cysteine-penicillamine disulfides which closely resemble cystine in molecular size, weight, and structure adds strong support to this view. The mixed disulfides have molecular weights of 269 and on the basis of size alone should be vacuolizing agents. That they do not vacuolize normal cells implies the presence of a physiological mechanism for their metabolism within, or transport from, the lysosomes.

Human cystinotic cells should be deficient in this activity, and the vacuolation of cystinotic cells by the mixed disulfides and their excessive accumulation of L-cysteine-D-penicillamine disulfide are entirely consonant with this view.

The nature of the specific gene product which appears to be deficient in cystinotic lysosomes requires further clarification. The experimental evidence presented here suggests that this gene product will be active with both L- and D-cystine, since L- and D-cysteine disulfides do not induce vacuolation of normal cells. The data also suggest that it will be inactive toward D-penicillamine disulfide and L-penicillamine disulfide, since these disulfides cause pronounced vacuolation of both cystinotic and normal lysosomes.

Any lysosomal mechanism for reducing cystine to the much smaller and presumably more permeable cysteine (mol wt 121) could account for lack of cystine accumulation in normal lysosomes. Little is known however about disulfide reduction or other aspects of disulfide metabolism in lysosomes. Peake and associates noted that (reduced) glutathione enhances proteolysis by thyroid lysosomes, and suggested that one possible mechanism by which it might
do so is to undergo disulfide exchange reactions with the cystine bridges of the protein (34). The physiological significance of this observation is unclear, and the exchange reactions with cystine could be nonenzymatic. There is some evidence that lysosomes of the rat intestinal epithelium contain a cystine-reducing enzyme, cystine-glutathione transhydrogenase, which is also present in the soluble cell fraction (35). Our own investigations however provide little support so far for the view that cystine-glutathione transhydrogenase plays a physiologically significant role in lysosomal disulfide metabolism in cystinotic (30) or normal white cells, particularly at the acid pH presumed to exist within lysosomes and typical of the pH optimum of nearly all lysosomal enzymes (13, footnote 2).

A transport system for cystine in the lysosomal membrane remains a significant theoretical possibility. Lucy has suggested that the processes involved in removing certain low molecular weight substances from lysosomes could involve the active transport of amino acids, sugars, and ions (33). Although no such mechanisms have been defined, the low internal pH of lysosomes may well be maintained by an active transport system for hydrogen ions. Furthermore, ATPase activity, which may be associated with membrane transport systems, has been reported in lysosomal particles (36, 37).

While the basic mechanism remains elusive, the studies of disulfide-induced vacuolation reported here provide rather strong confirmatory evidence that cystinosis is a lysosomal storage disease and provide some insight into the properties of the lysosome which plays a role in such storage. Furthermore, the selective induction of vacuolation in cystinotic fibroblasts by the conveniently synthesized L-cysteine-D-penicillamine disulfide provides the first histological marker for these genetically abnormal cells. This marker may prove useful in further investigations of cystinosis. Selective vacuolation might be applied toward the diagnosis of cystinosis using a very small number of cells, but the undefined variables which affect vacuolation in cultured cells indicate the need for extreme caution should this be attempted. We feel that the diagnosis of cystinosis by the specific measurement of intracellular cystine (28, 38) is, whenever feasible, greatly preferable to the vacuolation technique.

The demonstration that the permeability of human lysosomes may be conveniently investigated in genetically normal and abnormal skin fibroblasts, which are readily accessible and easily cultured, may encourage investigation of hitherto unstudied aspects of lysosomal metabolism in a number of genetic diseases of man.

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SUMMARY

The metabolism of amino acids, peptides, and disulfides has been investigated in cultured skin fibroblasts from normal individuals and patients with cystinosis. Human fibroblast lysosomes closely resemble the lysosomes of mouse peritoneal macrophages in having an apparent permeability barrier to amino acids and peptides with molecular weights of greater than 220-230. Cystinotic and normal cells behave similarly in this regard. Normal cells do not undergo lysosomal swelling when exposed to cysteine-penicillamine disulfides, while cystinotic cells are prominently vacuolized under these conditions. Normal lysosomes may have a specific mechanism for the disposal of cystine, and deficient activity of this mechanism in cystinotic lysosomes could result in cystine storage therein. The demonstration that human fibroblasts can be used conveniently to study lysosomal metabolism of small substrates may facilitate investigations of these aspects of lysosomal function in a variety of genetic diseases of man.

We thank Doctors L. Laster and J. E. Seegmiller for facilitating these investigations.

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